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**ADVANCES IN ENZYMOLOGY**

**AND RELATED SUBJECTS OF  
BIOCHEMISTRY**

**Volume IX**

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# **ADVANCES IN ENZYMOLOGY**

***AND RELATED SUBJECTS OF BIOCHEMISTRY***

**Edited by F. F. NORD**

**FORDHAM UNIVERSITY, NEW YORK, N. Y.**

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## SOME ASPECTS OF REVERSIBLE STEP REACTIONS

By L. MICHAELIS, *New York, N. Y.*

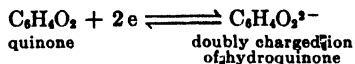
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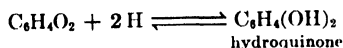
## I. The Problem

The theory of reversible step reactions has by now become a large field, although its literature is still widely scattered. Citations in the bibliography marked with an asterisk represent a list, although not a complete one, of the more important contributions. Out of the whole material a few topics will be selected here which have been of interest to enzymologists, but have not been fully appreciated by most of them.

Let us consider the reaction in which one molecule of the species A can combine reversibly with several molecules of the species B, their maximum number being  $N$ . The molecular species B will be referred to as the "ligand." The following examples will point out the occurrence of such reactions. First of all, there are the polyvalent acids. One polyvalent anion of a polycarboxylic acid can combine with several protons. Furthermore, a metal ion may form the center of a co-ordinative complex such as the cobaltihexammine ion, in which six equal ligands such as  $\text{NH}_3$  molecules are bound to one central metal ion (6). Furthermore, the reversible oxidation of organic compounds is usually a bivalent one, for instance:



or, when the reaction takes place in an acid solution:



The bivalent oxidation, provided it takes place in a homogeneous solution by the interaction of an oxidizable substance and an oxidizing agent due to molecular collisions, has been shown to take place in two successive univalent steps, which may overlap more or less (25).

More examples are encountered in the field of the hemochromogens. According to circumstances, in solution they may be molecularly dispersed, or contain bimolecular aggregates, or even poly-molecular micelles. Certain studies pertaining to this problem (37) arrived at the result, at the time unexplainable, that in a case in which the existence of dimeric molecular units was established from spectrophotometric data, the oxidation-reduction potential curve was the same as though the hemochromogen were present in homogeneous, molecularly dispersed solution. Other cases in this field have been described and discussed by Clark (12). Another example is the case of hemoglobin, whose molecule is a compound of a protein with four heme groups. This complex molecule can combine reversibly with four molecules of oxygen.

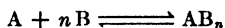
Another example may be taken from the field of enzymology. Suppose one molecule of an enzyme, E, say, invertase, combines with one molecule of the substrate, S (which is sucrose), to form a compound, ES, which has the property of decomposing spontaneously through its interaction with one molecule of water into the enzymic split products and free enzyme, at a rate much slower than that at which the equilibrium,  $\text{E} + \text{S} \rightleftharpoons \text{ES}$ , is established. This assumption was the basis of a theory of enzyme kinetics (18,26) established many years ago and since applied to several other enzyme reactions. A recent list of those enzymes to which this theory has been applied can be found in Baldwin (4). The quantitative aspect of this theory and the interpretation of the "affinity constant" as derived from that theory is, seemingly, obscured by the fact that an enzyme is present in many cases, if not always, not as a homogeneous solution of the molecularly dispersed enzyme molecules, but rather in the form of colloidal aggregates or micelles, each of which has several active groups which can react with the substrate. In the case of invertase, the enzyme may even be entirely adsorbed

on macroscopic particles of ferric hydroxide or aluminum hydroxide (23) in such a way that after centrifugation the supernatant is entirely free from enzyme, and yet the suspension acts on saccharose just as does the "free" enzyme. Neuberg has prepared invertase also in the form of an insoluble calcium phosphate adsorbate (30). Some authors, for this reason, prefer to speak of an "adsorption" of the substrate at the "surface" of the enzymic micelle and even emphasize the difference between such an "adsorption compound" and a true "chemical" compound. I do not wish to reiterate the whole discussion which has not ceased even now, but should rather like to refer the reader to an old paper (24) to which nothing essential can now be added.

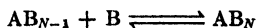
In still other cases the enzymic activity is inherent entirely in the surface of larger particles or granules, or "mitochondria," e.g., in the case of Warburg's respiratory enzyme or of succinic dehydrogenase. In all such cases one enzymic micelle or aggregate, or macromolecule, is supposed to be able to adsorb or react with several molecules of the substrate. The simple mass action law, as originally used with the idea in mind that the enzyme is molecularly dispersed, has been deemed by some authors to be inadequate for the theory which correlates a certain concentration of the substrate (namely the concentration at which the rate of enzymic action is one-half the maximum rate) to the "affinity constant" of the enzyme-substrate compound (26).

## II. Theory of Step Reactions

If a molecule, A, can combine with more than one molecule of a ligand, B, forming the compound  $AB_n$ :



there is extremely little chance that such a reaction may take place by a simultaneous collision of one molecule of A with  $n$  molecules of B. Rather the only reasonable chance available is a step reaction, each step being a bimolecular reaction:



$N$  being the maximum number of B molecules able to combine with one molecule of A. For each step an individual equilibrium constant is valid. If the successive constants differ greatly, the intermediate complexes may be separated or may at least be readily recognized as individual molecular species. If the successive equilibrium constants are of a magnitude more comparable to each other, the steps will overlap and the identification of intermediary products may be difficult or even practically impossible.

First of all, let us recall the significance of a single equilibrium constant as it can be derived from kinetic (instead of thermodynamic) considerations. Although nothing new can be added here, the writer wishes to recapitulate the arguments briefly because of their fundamental importance to what follows. Equilibrium in reaction (1) is reached when the rate of the formation of AB from A and B equals the rate of its dissociation into  $A + B$ . The rate from left to right in reaction (1) is determined by a binary collision. This rate,  $R_{\rightarrow}$ , is, therefore,

$$R_{\rightarrow} = c_2[A][B]$$

where  $c_2$  is a proportionality factor representing a constant characteristic for this particular reaction. It is designated  $c_2$  in order to indicate that it refers to a bimolecular reaction. The rate from right to left is a spontaneous unimolecular process. The rate  $R_{\leftarrow}$  is:

$$R_{\leftarrow} = c_1[AB]$$

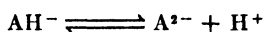
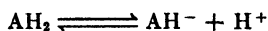
where  $c_1$  is the proportionality factor characteristic of this unimolecular reaction and  $[AB]$  is the concentration of the compound AB. In the state of equilibrium,  $R_{\rightarrow} = R_{\leftarrow}$  or:

$$\frac{c_1}{c_2} \times \frac{[A][B]}{[AB]} = 1$$

and: 
$$\frac{[A][B]}{[AB]} = \frac{c_2}{c_1} = k \quad (4)$$

The ratio  $c_2/c_1$  is obviously the dissociation constant,  $k$ , of the compound AB. The reciprocal of  $k$  may be called the association constant or the affinity constant.

Now let us consider a simple case of a two step reaction, illustrated by a scheme according to Adams (1). The bivalent acid  $AH_2$  can dissociate in these two steps;

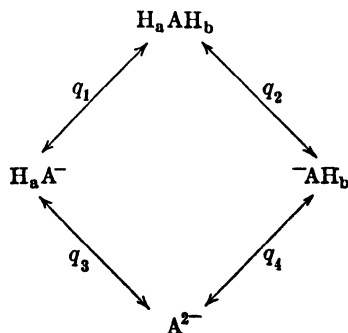


The two dissociation constants are:

$$k_1 = \frac{[\text{AH}^-][\text{H}^+]}{[\text{AH}_2]}$$

$$k_2 = \frac{[\text{A}^{2-}][\text{H}^+]}{[\text{AH}^-]}$$

Now, the ion  $\text{AH}^-$  can arise in two different ways. If we distinguish the two H atoms as  $\text{H}_a$  and  $\text{H}_b$ , the ions represented as  $\text{AH}^-$  are a mixture of  $\text{AH}_a^-$  and  $\text{AH}_b^-$ . In some cases the two ions can be readily distinguished; for instance, there may be one proton and one deuteron. Or if the acid has an asymmetric structure, such as  $\text{HOCC}(\text{R}_1)\text{C}(\text{R}_2)\text{COOH}$ , and even if the two ionic species cannot be distinguished, as in  $\text{COOHCOO}^-$  and  $\text{COO-COOH}$ , there



SCHEME I

still remains the fact that the chance of establishing the ion from the undissociated acid is double that of an ordinary case. With these ideas in mind, the following scheme represents the dissociation of a bivalent acid (Scheme I). Each step is characterized by a specific dissociation constant,  $q_1$ ,  $q_2$ ,  $q_3$ , and  $q_4$ . The definition of these four constants is:

$$q_1 = \frac{[\text{H}_a\text{A}^-][\text{H}^+]}{[\text{H}_a\text{AH}_b]}$$



$$\begin{aligned}
 q_2 &= \frac{[\text{AH}_b][\text{H}^+]}{[\text{H}_a\text{AH}_b]} \\
 q_3 &= \frac{[\text{A}^{2-}][\text{H}^+]}{[\text{H}_a\text{A}^-]} \quad \text{or} \quad \frac{1}{q_3} = \frac{[\text{H}_a\text{A}^-]}{[\text{A}^{2-}][\text{H}^+]} \\
 q_4 &= \frac{[\text{A}^{2-}][\text{H}^+]}{[-\text{AH}_b]} \quad \text{or} \quad \frac{1}{q_4} = \frac{[-\text{AH}_b]}{[\text{A}^{2-}][\text{H}^+]}
 \end{aligned}$$

In an ordinary titration experiment we cannot distinguish the two kinds of univalent ions. What appears to be the ion  $\text{AH}^-$ , is in reality a mixture of  $\text{H}_a\text{A}^-$  and  $-\text{AH}_b$ . Therefore, the dissociation constants as determined from an electrometric titration curve are:

$$k_1 = \frac{[\text{AH}^-][\text{H}^+]}{[\text{AH}_2]} = \frac{([\text{H}_a\text{A}^-] + [-\text{AH}_b])[\text{H}^+]}{[\text{H}_a\text{AH}_b]} = q_1 + q_2 \quad (5)$$

Here  $k_1$  is the dissociation constant for the first step, and:

$$\frac{1}{k_2} = \frac{[\text{AH}^-]}{[\text{A}^{2-}][\text{H}^+]} = \frac{[\text{H}_a\text{A}^-] + [-\text{AH}_b]}{[\text{A}^{2-}][\text{H}^+]} = \frac{1}{q_3} + \frac{1}{q_4} \quad (6)$$

where  $1/k_2$  is the association constant for the second step. The dissociation constant itself,  $k_2$ , is:

$$k_2 = \frac{1}{1/q_3 + 1/q_4} = \frac{q_3 q_4}{q_3 + q_4}$$

The constants  $k$  and  $q$  are sometimes distinguished as macroscopic and microscopic constants. We shall here refer to the  $q$  values as the *fundamental* constants, and to the  $k$  values as the *practical* constants.

Now let us consider the special case that the chemical nature of the two groups capable of dissociation is the same, and that the ejection of one proton provides neither inhibition nor enhancement for the ejection of another proton. This is the case when the two carboxyl groups are separated by a very long carbon chain, which makes the electrostatic coulomb forces negligibly small. This holds especially also for the case in which the dissociation is not an electrolytic one and the dissociation products are electroneutral, or where no change in the number of free electrical charges is involved. It may hold for the case of a large enzymic micelle, where the active

groups are widely separated and, in general, no essential electrostatic forces of the coulomb type, decreasing with the second power of the distance, may be expected. In such a case  $q_1 = q_2 = q_3 = q_4$ , and:

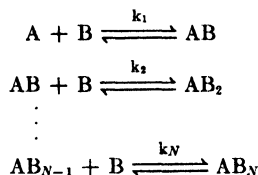
$$k_1 = 2q$$

$$k_2 = q/2$$

In words: the ratio of the two practical constants,  $k_1/k_2$ , is 4/1; and the geometric mean value of the two practical constants is the fundamental constant:

$$k = \sqrt{k_1 k_2} = q$$

A more general consideration is this: consider the step reaction:



$N$  being the maximum number of B capable of combining with one A each. The general case is:



where  $n$  is any integer smaller than  $N$ . The molecular species  $AB_{n+1}$  has  $(n+1)$  times the chance to eject a molecule of B than would correspond to a fundamental dissociation constant. On the other hand, the species  $AB_n$  has  $(N-n)$  co-ordination places unoccupied and therefore an  $(N-n)$  times greater chance to combine with one molecule of B than would correspond to a fundamental association constant. So, the practical dissociation constant of the process will be  $(n+1)/(N-n) \times q$ , where  $q$  is the fundamental dissociation constant. Setting  $n$  equal to 0, 1, 2 . . .  $n$  . . .  $N$ , one obtains, for the successive practical dissociation constants  $k_1, k_2$  . . .  $k_n$  . . .  $k_N$ , always under the supposition that all fundamental dissociation constants are alike ( $= q$ ), the values of Table I.

Now one may inquire about the average value of the  $k$  terms. Such a problem arises in this way: When, for instance, a dibasic acid is titrated with sodium hydroxide and pH plotted against the

TABLE I<sup>a</sup>  
 SUCCESSIVE PRACTICAL DISSOCIATION CONSTANTS IN FORMS  
 OF THE FUNDAMENTAL DISSOCIATION CONSTANT

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$\frac{[A][B]}{[AB]}$	$= k_1 = q \frac{N}{1}$
$\frac{[AB][B]}{[AB_2]}$	$= k_2 = q \frac{N-1}{2}$
$\frac{[AB_2][B]}{[AB_3]}$	$= k_3 = q \frac{N-2}{3}$
$\vdots$	
$\frac{[AB_{n-1}][B]}{[AB_n]}$	$= k_n = q \frac{N-n+1}{n}$
	$k_{N-1} = q \frac{2}{N-1}$
	$k_N = q \frac{1}{N}$

---

<sup>a</sup> It may be added: If  $N$  is odd, say  $N = 2m + 1$ , there is a middle term,  $k_{m+1} = q \frac{(2m+1) - (m+1) + 1}{m+1} = q$ . If  $N$  is even, there are two middle terms, one with a factor very slightly greater than 1, the other very slightly less than 1, the average being equal to 1; and, if  $N$  is large both are practically equal to 1.

alkali added, and if the two successive dissociation constants differ greatly (their ratio  $\gg 1000$ , say), the titration curve consists of two sections, each representing the sigmoid titration curve characteristic of a univalent acid. The two moieties of the curve are connected by a steep rise between the two flatter ("buffered") parts of the curve. As the ratio of the two successive constants becomes smaller, this rise becomes less conspicuous, and may show up in the form of a just noticeable steepening in the middle of the curve. As the ratio of the two constants becomes 16/1, this steepening disappears altogether (2,25), and the whole curve has the same shape as for a univalent acid, except for the fact that its slope is steeper. As the ratio of the constants becomes as small as 4/1, which is the smallest ratio possible, the curve is entirely undistinguishable, also with respect to its slope, from a univalent titration curve. A univalent acid of concentration  $[a]$  would give, in this case, the same

titration curve as a bivalent acid of molar concentration 0.5  $[a]$ . The  $pK$  of the univalent acid equals the  $pH$  in the middle of the curve. For the case of the bivalent acid, the corresponding point may be called the mean, or average,  $\overline{pk}$  of the bivalent acid:

$$\overline{pk} = (pk_1 + pk_2)/2$$

If one wants to deal with the dissociation constants themselves, instead of with their (negative) logarithms, one has to take the geometric instead of the arithmetic mean:

$$\bar{k} = \sqrt{k_1 k_2}$$

In analogy to this simple case it will be of interest to inquire about the geometric mean value  $\bar{k}$  of all the various  $k$  terms in Table I, which is by definition:

$$\bar{k} = (k_1 k_2 \dots k_{N-1} k_N)^{1/N}$$

Now, on considering the factors of  $q$  in the third column of Table I, the product of this factor in the first term in this column by that in the last is unity, and so is the product of the second by the last but one, etc. Thus if  $N$  is an even number:

$$\bar{k} = q$$

In words, *the average value of the practical constants is the fundamental constant*. If  $N$  is odd, one has to remember that the factor  $q$  in the middle term itself is unity, so this result is quite general.

Let us now consider the equilibrium established between (1) the molecules of species B combined with the large molecule, "macro-molecule" or "micelle," A, and (2) the free molecules of B. In order to calculate the conditions for equilibrium, we first calculate the rate at which molecules of the kind B are being bound, per unit time, to molecules of the kind A, as a possible consequence of a binary collision. If all co-ordination places of all A were unoccupied, this rate would be  $c' [A][B]$ , where  $[A]$  is the concentration of A,  $[B]$  the concentration of B, and  $c'$  is a proportionality factor characteristic for this particular bimolecular reaction. If of the  $N$  co-ordination places\* of A, only the fraction  $\alpha$  is free, and if, of all

\*  $N$  is here the number of all the co-ordination places in all the A molecules, together, of the system. If of all these  $N$  places  $n$  are occupied, it does not mean that there is a solution of one single molecular species  $AB_n$ , but rather may there be a mixture of A, AB,  $AB_2$ ,  $AB_3$ , etc., such that the total number of places occupied by B, is  $n$ .

the molecules of B, only the fraction  $\beta$  is free in solution and the remainder, the fraction  $(1 - \beta)$ , is bound to A, the rate  $R_{\rightarrow}$  would be  $c'a[A]\beta[B]$ . On the other hand, we calculate the rate at which  $AB_n$  dissociates into  $AB_{n-1} + B$ . If all  $N$  co-ordination places of all A molecules together were occupied by B molecules, the rate of dissociation would be  $c''[A]$ . Here,  $c''$  is a proportionality factor characteristic of this reaction, and  $[A]$  is the concentration of A. If, now, only the fraction  $\alpha$  of all co-ordination places is free, and so the fraction  $(1 - \alpha)$  is occupied, the rate of dissociation is:

$$R_{\leftarrow} = c''(1 - \alpha)[A]$$

Equating  $R_{\rightarrow}$  and  $R_{\leftarrow}$  one obtains as condition for equilibrium:

$$c'a[A]\beta[B] = c''(1 - \alpha)[A]$$

or:

$$\alpha = \frac{c''}{c'' + (c'\beta B)} = \frac{c''/c'}{(c''/c') + \beta B} \quad (7)$$

Now the ratio  $c''/c'$  must be related to a dissociation constant in analogy to equation (4). However the analogy does not go so far that one could immediately tell what this relation is and which dissociation is meant, for the following reason: In the former, simpler case we had to deal with a system consisting only of the two molecular species A and B, and the compound formed from them. Here, however, we have to deal with an equilibrium mixture of all the species, A, AB,  $AB_2$  . . .  $AB_N$ . There are various ways to give the correct answer to the question as to which dissociation constant is meant by  $c''/c'$ . The simplest way to answer this question seems to be this: As one titrates A with B, one obtains a titration curve which may be imagined to arise from the overlapping of the successive titrations for the single steps  $A \rightarrow AB$ ,  $AB \rightarrow AB_2$ , . . .  $AB_{N-1} \rightarrow AB_N$ . At that point of titration at which A is combined with 50% of the maximum number of B molecules, the curve represents the titration of the middle step. The midpoint of this section is indicative for the dissociation constant of the middle step. Now, it had been shown that, for the middle step, the practical constant is the same as the fundamental constant. So, the midpoint of the whole titration curve is indicative for the fundamental constant of the system. Since equation (7) is valid in general, it must

hold also for the middle section of the curve. Herefrom it can be seen that  $c''/c'$  is not only proportional to, but even is, the fundamental dissociation constant itself.

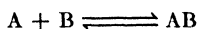
The idea may be stated also as follows: When half the maximum number of B is attached to A, or, in other words, when the number of free and of occupied co-ordination places is the same, the statistical factor for dissociation and for association is the same, and so cancels out. Therefore, putting  $c''/c' = q$ , we obtain:

$$\alpha = q/(\beta[B] + q) \quad (8)$$

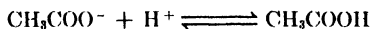
It should be remembered that  $\alpha$  is the fraction of all co-ordination places of A which are free and not bound to a molecule B; and  $\beta$  is the fraction of all B molecules free in solution and not bound to A.

These considerations are based on the same fundamental principles used by Langmuir (20) in the development of his equation for adsorption equilibrium. In fact, the two cases are of the same nature and may be quite indistinguishable. The continuity of the mass action law as applied to homogeneous solutions with the theory of adsorption as applied to colloidal solutions or suspensions of one phase in another phase was emphasized also by Linderstrøm-Lang (21).

This rule must hold for any value of  $N$ , so also for  $N = 1$ , i.e., for a univalent acid, or in general for the simple case:



Let us take an example. Add to a solution of acetate ions (in the form of sodium acetate), which stand for what was designated as A, a certain amount of  $H^+$  ions (in the form of hydrochloric acid), which represents B. Then, a part of the acetate ions combines with  $H^+$  ions:



Applying equation (8) we have to put, in this case:

$$\alpha = \frac{[\text{total A}] - [AB]}{[\text{total A}]} = \frac{[CH_3COO^-]}{[CH_3COO^-] + [CH_3COOH]}$$

and:

$$\beta = \frac{[\text{free B}]}{[\text{total B}]} = \frac{[H^+]}{[HCl]}$$

where  $[HCl]$  represents  $[\text{total B}]$ . Hence,  $\beta[B] = [H^+]$ . Thus equation (8) reads in this case:

$$\frac{[\text{CH}_3\text{COO}^-]}{[\text{CH}_3\text{COOH}] + [\text{CH}_3\text{COO}^-]} = \frac{q}{q + [\text{H}^+]} = \frac{k}{k + [\text{H}^+]} \quad (9)$$

For a univalent A, no distinction can be made between  $k$  and  $q$ . This formula is very familiar and could have been arrived at in a much simpler way. For it represents the expression for the degree of dissociation,  $a$ , of the acid:

$$a = k/(k + [\text{H}^+]) \quad (10)$$

We see, that  $q$  in equation (9) is the dissociation constant as shown in equation (10). For instance, when  $a = 0.5$ ,  $k = [\text{H}^+]$ . This is the familiar way to determine the dissociation constant of a simple weak acid or base.

It may be recalled that the dissociation residue,  $\rho$ , equals  $1 - a$ ; thus:

$$\rho = [\text{H}^+]/(k + [\text{H}^+])$$

This function will be used later on.

Quite generally, on titrating a molecular species, A, with the molecular species, B, with which it can combine, *the titration curve does not permit one to distinguish whether a molecularly dispersed solution of A has been titrated, or whether a molecular aggregate, or a micelle, possessing many groups or sites able to combine, each with one molecule of B, has been titrated.* This fact seems to have been recognized first by Pauli and Valkó (32). If A is molecularly dispersed, the midpoint of the titration curve, where one-half the amount B capable of combining maximally with A is combined with A, indicates the dissociation constant, there being no distinction necessary between a practical and a fundamental one. If A is a polyvalent aggregate, the same method reveals the "fundamental" dissociation constant. Its reciprocal is the association constant, or affinity constant.

In the case of a polyvalent A, there is a whole series of practical dissociation constants, one for each step. If, according to our supposition, we are dealing with cases in which these constants are all in the statistical ratio as in Table I, there is only one common value for the fundamental constant,  $q$ . As has been shown the practical constant in the middle of the titration curve (50% saturation of A with B) equals the fundamental constant, and is a true measure for the intrinsic affinity of A for B.

If one is not sure whether it is true that there is only one funda-

mental constant, one need just compare also some points other than the midpoint of the titration curve, with the corresponding ones of a curve for univalent A. Whenever the two curves coincide there is only one fundamental constant, and one can speak of one definite affinity constant for the attraction of B to any co-ordination place of A. When, however, the curve is steeper than that expected for a univalent one, and especially if the curve is "wavy," instead of being strictly sigmoid, this is evidence for the fact that the successive dissociation constants are not in the statistical ratio and that there is not one fundamental constant but that the attachment of one molecule B to one co-ordination place of A influences (usually, but not always, diminishes) the affinity of another molecule of B to combine with another co-ordination place of A.

Two examples will be discussed for the latter condition. If one "titrates" hemoglobin with oxygen and plots the concentration (or pressure) of free oxygen against bound oxygen, the titration would be a parabola if the hemoglobin molecule contains one heme capable of binding one oxygen molecule. This situation is encountered for myoglobin (28). For hemoglobin, with its four heme groups, each capable of binding one oxygen molecule, the curve is different. It can be inferred herefrom that the four fundamental affinity constants are not alike, but that the affinity for one oxygen molecule is different whether it is the first to be attached or the second, etc. In this particular case the attachment of one oxygen molecule facilitates the attachment of another oxygen molecule, which is remarkable indeed. Pauling (33) assumes that the influence of one oxygen molecule upon another depends on the distance between them, and, postulating an arrangement of the four hemes in a square, that the magnitude of influence of one oxygen molecule upon another depends on whether the two oxygen molecules considered are neighbors or lie on a diagonal. Wyman (43) arrives at a somewhat different interpretation. The whole problem needs further investigation, since Barron has shown (5) that the shape of the curve depends largely on the ionic strength of the solution.

Numerous other examples in which the fundamental constants of the successive steps are not alike and therefore the practical constants are not in the statistical ratio are the polyvalent acids. After one proton has been ejected, the negative charge created thereby exerts an attraction for another proton which counteracts



its tendency of dissociation. This is a purely electrostatic influence. Its magnitude depends essentially on the distance, within the molecule, of the two places of dissociation concerned. N. Bjerrum (8) succeeded in calculating with reasonable accuracy this electrostatic effect due to coulomb forces. Later J. Bjerrum (7) showed that in the majority of cases, in the reversible dissociation of the heavy metal complexes of the Werner type (such as cobaltihexammine), effects other than electrostatic ones are in most cases very small, if there are any at all.

An especially important case of this kind is encountered when a protein is titrated with a strong acid or base. The macromolecule of the protein contains many groups capable of combining with a proton, some of them of chemically equal nature, others of different chemical character; one kind may be represented by the  $\text{NH}_2$  groups, others by the  $\text{COO}^-$  groups. Here the problem arises whether there is one fundamental dissociation constant for all chemically equivalent  $\text{NH}_2$  groups, or whether the dissociation of the first amino group electrostatically influences the dissociation constant for the next one. This kind of problem is of especial interest for protein chemistry. It is not the intention of this review to discuss it in detail. Reference to the papers by Linderstrøm-Lang (21), and to the quotation of a personal communication by Wyman, in a paper by Edsall must suffice (42). However, we may summarize as follows the various possibilities:

(a) The affinity of one co-ordination place to a ligand may be quite independent of whether another co-ordination place is already combined with a ligand molecule. In this case the ratio of the practical dissociation constants is the statistical one. This situation can be expected to be encountered especially if B is an electroneutral molecule or if the dissociation neither creates nor destroys any free electric charges. One may speak here of one common fundamental dissociation constant, or one single affinity constant.

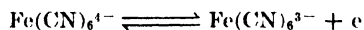
(b) Whenever electrostatic effects must be taken into consideration, the ratio of each two successive dissociation constants is greater than the statistical one.

(c) Even without the interference of simple electrostatic forces of the simple coulomb type, it sometimes may not be true that the dissociation of one molecule of the ligand has no influence at all on the dissociation of a second ligand molecule, as in the example of

oxyhemoglobin. Such influences, however, if they exist, are usually small. These effects may be due to various, partly known and partly unknown factors and may be summarized as "rest effect" (6). It may be interesting even in this review to mention J. Bjerrum's result, holding for inorganic metal complex compounds, namely, that, in the absence of electrostatic effects, or if they exist, after correction of such electrostatic effects, the successive practical dissociation constants of a complex such as, for example, of the type  $\text{Co}^{\text{III}}(\text{NH}_3)_6^{3+}$ , with respect to the dissociation of the six ligand molecules, are usually very nearly in the statistical ratio. Then one can determine with fair accuracy (and speak of) one fundamental constant common to each step of dissociation.

#### A. REVERSIBLE REDOX SYSTEMS FORMALLY REPRESENTED AS DISSOCIATION SYSTEMS

The whole field of *reversible oxidation* can also be dealt with as another example of dissociation. The reduced form may be said (11) to be able to dissociate into (1) the oxidized form, and (2) an electron. This electron need not be free in solution, but it may be combined with an "electron acceptor," i.e., an "oxidizable substance," just as, in an acidic dissociation, the proton which arises from the dissociation of the acid is present combined with a proton acceptor such as water. The "acceptor" when combined with the electron (or proton as the case may be), exhibits a finite, measurable tendency to split out an electron (or proton) and so exerts a definite "pressure" or "activity" of electrons (or protons). Such a dissociation may be formulated thus:



The mass action law, then, defines the equilibrium as follows:

$$\frac{[\text{Fe}(\text{CN})_6^{3-}][e]}{[\text{Fe}(\text{CN})_6^{4-}]} = k$$

or:

$$\log [e] = \log k - \log \frac{[\text{Fe}(\text{CN})_6^{3-}]}{[\text{Fe}(\text{CN})_6^{4-}]}$$

It can be seen herefrom that the relation of  $[e]$  and the oxidation potential  $E$  is this:

$$(RT/F) \log [e] = E$$

Let us now consider an enzyme reaction under the following experimental conditions: In a series of independent experiments the

amount of the enzyme, E (say invertase), is the same, and the concentration of the substrate, S (sucrose), is varied. The reversible reaction:



takes place, which, read from right to left, represents the dissociation of the compound ES. The concentration of this compound is supposed to be proportional to the initial rate of enzymic splitting. Thus, the ratio  $[ES]/[\text{total } E]$  or the dissociation residue,  $\rho$ , is proportional to the initial rate of enzyme action. The dissociation residue,  $\rho$ , is analogous to that of the reaction:



where:

$$\rho = \frac{[CH_3COOH]}{[\text{total acid}]} = \frac{[CH_3COOH]}{[CH_3COOH] + [CH_3COO^-]} = \frac{[H^+]}{[k] + [H^+]}$$

The difference between the two cases is essentially quantitative, not qualitative. The dissociation constant of ES is very much larger than that of  $CH_3COOH$ . In equation (14)  $H^+$  is analogous to S in equation (13). Now it is known for the case of an acid that, on plotting  $\rho$  against  $\log [H^+]$ , one obtains a sigmoid curve, and  $\log [H^+]$  in the midpoint equals  $\log k$ . Analogously, if we plot  $\rho = [ES]/[\text{total } E]$  against  $\log [S]$ , one obtains a sigmoid curve, and  $\log [S]$  in the midpoint equals the log of the dissociation constant,  $k$ , of reaction (13). Since  $[\text{total } E]$  is being kept constant,  $[ES]$  plotted against  $[S]$  will yield the same type of curve, differing only in the scale of the ordinate, and thus also the initial enzymic rate of reaction plotted against  $\log [S]$  gives a sigmoid curve;  $\log [S]$  in its midpoint equals the dissociation constant of ES, and  $-\log [S]$  equals its reciprocal, namely, the affinity constant of enzyme to substrate.

If now the enzyme is present in the form of molecular aggregates or particles containing at its surface several enzyme sites, we have to apply again the theory of step reactions as follows: In the same manner as in the simple case of a one step reduction just discussed, the midpoint of the curve indicates  $pK$ , so also the midpoint of the many step reaction indicates a dissociation exponent. In the simple one step case there can be no doubt which dissociation constant is meant because there is just one. In the  $N$  step reaction there are

many constants. There is not one, but many simultaneous dissociation processes to be considered since there is always a mixture of A, AB, AB<sub>2</sub> . . . AB<sub>N</sub>. It has been shown previously that, whenever the practical constants are in the statistical ratio, the constant corresponding to the midpoint of the sigmoid curve equals the fundamental constant, and there is only this one fundamental constant. So, whenever a many step titration curve agrees both in shape and in slope with a one step curve, this is not only evidence for the fact that all successive constants are in the statistical ratio, but also for the fact that the dissociation constant obtained graphically as though it were a simple one step reaction is the fundamental constant of the many step reaction. Its reciprocal represents a rational measure for the affinity of the ligand B to the macromolecule A to which it can be bound.

On the other hand, whenever the titration curve is not symmetrical about its midpoint and, even though symmetrical, has a slope steeper than expected for a univalent titration curve, one may infer that the successive practical dissociation constants are not in the statistical ratio, and that the fixation of one B to A influences the affinity with which another B is fixed to another co-ordination place of the polyvalent B. If the curve should be symmetrical about its midpoint, but steeper than a one step curve, the midpoint of the titration is at least indicative of an average value of the fundamental constants,  $\bar{q}$ .

If one applies this consideration to the theory of enzyme kinetics and makes the assumptions stated above, one may say that for the case of invertase, and for several other enzymes,\* the "dissociation curve" has a shape, within the limits of experimental error, to justify the existence of one definite affinity constant. The limitations of the experimental evidence are these: In order to determine, for a given amount of enzyme and with varied substrate concentrations, the asymptotic maximum rate of decomposition, one has sometimes to employ so high a concentration of the substrate that it becomes doubtful whether the simple mass action law, with concentrations instead of activities, is still valid, since the concentration of water or, better, its activity as measured by its vapor pressure, is no longer the same as for low concentrations. In the

\* Recently another case has been added by Egami and Sato (14) for the "nitrate reductase" of *Escherichia coli*. This enzyme can be extracted only by ultrasonic vibrations and is obviously not molecularly dispersed.

case of invertase, the maximum rate is asymptotically reached at a sugar concentration so high that the solution can no longer be considered as a simple "aqueous solution." In fact, with increasing concentration of substrate there is no asymptotic maximum of the rate established; rather does the rate pass through a maximum, after which it begins to decline slightly (with emphasis on the word "slightly"). There is, then, a slight uncertainty with respect to the true theoretical maximum rate. This, however, is not a serious matter. The maximum rate can be extrapolated to a degree of safety quite sufficient for the purpose. Nobody will expect the affinity constant to be determined within, say,  $\pm 1\%$ . The whole procedure, then, is this: The initial enzyme reaction rate is plotted against the logarithm of the sucrose concentration. The theory requires that this rate be proportional to the concentration of the ES compound, but the proportionality factor is unknown. By adjusting the scale of the ordinate so that the maximum rate corresponds to unity, the curve represents a dissociation residue curve. The proportionality factor, which "normalizes" the ordinate, is the only arbitrary constant occurring in the whole procedure. If this curve shows no waves, and if its slope is the one expected for a univalent one step dissociation, there is a choice between those two interpretations: (a) The enzyme is molecularly dispersed, and the enzyme-substrate compound, ES, has a dissociation exponent  $pK$  which equals the logarithm of the sucrose concentration at one half the maximum rate; or (b) The enzyme is present in the form of multimolecular micelles, and the combination of one sucrose molecule to one site of the micelle has no influence upon the affinity with which another sucrose molecule is bound to any other site of the micelle. The affinity constant, corresponding to the point at half-maximum rate is now the *fundamental* constant, which after all, is the true measure for the affinity of substrate and enzyme.

Whenever the dissociation residue curve obtained by the method has a slope steeper than corresponds to a one step process, one has to conclude that the enzyme is not molecularly dispersed and that the affinity of one molecule of substrate to a site of the micelle depends on whether or not another site of the micelle has already been occupied by a molecule of substrate. In this case, the result of this method is not a *fundamental affinity constant*, but just the *average* of the successive fundamental constants; in such a case

the successive practical constants are not in the statistical ratio shown in Table I.

### C. TWO SPECIAL CASES

In a few cases the initial velocity of the enzyme reaction does not have a maximum asymptotically reached with increasing concentration of substrate, but exhibits a distinct maximum and thereafter a sharp decline with further increase of substrate concentration in such a manner that the decline practically reaches the zero line at substrate concentrations that are not extremely high. This has been found to hold for the hydrolysis of acetylcholine by certain preparations of various choline esterases (3) and for the action of amino acid oxidase of *Vipera aspis* on its substrate (44). In such cases it is likely that the substrate molecule must be attached at two sites of the enzyme molecule in order to be attacked by it, and that with increasing concentration each substrate molecule may be forced to be satisfied with one site and thus will not be "activated" by the enzyme.

### D. SOME OBJECTIONS

In conclusion I wish to discuss some objections that have been raised against the fundamental assumptions underlying the theory of enzyme kinetics. The main objection (15,22,31) is that the same type of enzyme kinetics can be derived from the assumption that the interaction of enzyme and substrate takes place as a consequence of instantaneous molecular collisions without the formation of any enzyme-substrate compound. Such an assumption amounts to denying any attractive force between substrate and enzyme. In the field of other proteins on highly specific functions, the phenomenon of attractive forces is well known, for example, antibody-antigen attraction. It results in the demonstrable formation of a compound of the two. It is not farfetched to extend the idea of an attracting force to that other field of specific proteins, namely, the enzymes. However, even disregarding such an argument by analogy, even for the simplest bimolecular reactions the assumption of a kind of intermediate compound is quite customary in modern theories of chemical kinetics: The rate of chemical reactions depends on the frequency with which this compound dissociates again without the occurrence of interaction compared to the frequency at which the compound undergoes a rearrangement, with

the chemical reaction ensuing. The difference between the "collision theory" and the "compound theory" is but a quantitative one; the average lifetime of the "compound" may be short or long. Now, if the lifetime of the compound were extremely short and the enzyme reaction depended only on the frequency of collisions, it would be impossible to explain the competitive inhibition often exerted by other molecular species, especially the split products of the enzyme process. Why should fructose inhibit the action of invertase on sucrose, whereas lactose has no influence at all? How could one assume that fructose diminishes the frequency of collisions between enzyme and sucrose, whereas lactose does not? There obviously exists a compound of invertase with fructose (but not with lactose) which is reversible, to be sure, yet has a finite average lifetime. The existence of this compound explains why fructose distracts the enzyme from reacting with sucrose. In some cases (10,39) the existence of an enzyme-substrate compound has been proved by spectrophotometric methods. Although it need not be true that what holds for one enzyme must hold for any other, this observation is at least supporting evidence.

Northrop (31) describes a case in which the rate of hydrolysis for an enzyme on one protein (gelatin) is the same whether or not another protein (casein) is being hydrolyzed simultaneously. This fact would be a serious objection if one could be sure that it is the same enzyme, or at least the same enzymic site of the enzyme-protein molecule that acts on both hydrolyzable proteins. If one considers the high specificity of the various proteinases and peptidases with respect to minute details in the structure of the substrate, it is not farfetched to assume that the enzyme used is either a mixture, or, at least, a protein with two enzymic sites of different specificity.

Another objection of Northrop's is that the theory of enzyme kinetics uses several arbitrary constants and for this reason is not convincing. As a matter of fact, the theory uses no more than a single arbitrary constant, namely, the proportionality factor by means of which the scale of the ordinate is "normalized" so that its maximum value becomes equal to unity. Quite in contrast to the objection just mentioned, the essential feature of the theory is the very claim that a proper choice of this single factor makes the whole curve coincide with, or superimposable upon, a dissociation residue



curve, not only with respect to its resemblance in shape, but also with respect to its slope, point by point, within the limits of experimental error.

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# KINETICS OF BIOLOGICAL REACTIONS WITH SPECIAL REFERENCE TO ENZYMIC PROCESSES

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## I. Kinetics

Even in the simplest of systems any rationally satisfactory interpretation of kinetic data has lagged behind theoretical treatments in other fields. The lulling law of "initial and final states" cannot be invoked. The "same reaction" may vary not only in velocity but in apparent order depending on the path followed. In some quarters one encounters the sentiment that apparently the nice classification of reactions into first order, second order, etc. seems largely confined to the few particular examples invariably cited in textbooks. There have been two restrictions imposed, consciously or unconsciously, on kinetic investigations. The actual velocity of any reaction has had to lie between comparatively narrow limits to render it measurable. Also the reacting systems studied have been restricted to those which undergo a comparatively small number of

simultaneous reactions. This is necessary if relevant data are to be easily obtained.

In dealing with biological reactions, we frequently encounter a high degree of complexity, and it is difficult to simplify these processes in such a manner that we can be sure our simplified treatment has direct bearing on the unsimplified natural process.

A large part, possibly a great majority, of velocity studies are not utilizable for kinetic studies. In most of these cases the experiments were not designed for kinetic interpretation. Thus, the influence of various factors on the rates of vitamin loss in foods on storage or processing, on rates of permeation of undesirable foreign matter through protective coatings, and on corrosion rates, and the specific effects of preservatives or catalysts may be of great interest and value merely as empirical speed data.

In applying kinetics to systems involving biological materials there seem to be two general processes of interest: (1) reactions involving "biological" substances but in a nonliving condition, exemplified by inactivation of enzymes, catalytic action of enzymes *in vitro* on proper substrates, denaturation of proteins, etc., and (2) processes which are possibly more strictly biological since they take place in living organisms as a part of the "living" experience. Among the latter processes are to be found segmentation processes, regeneration processes, respiratory oxidations, etc. It is very tempting to think of, and to treat, many of these processes as ordinary chemical reactions. Such a procedure has the advantage of being a definite methodology, and frequently does throw suggestive light on the mechanisms of such processes.

## II. General Formulation of Reaction Rate Theory

The use of the Eyring formulation (22) of rate theory in studying kinetic data and planning kinetic experiments has several advantages. This formulation can be applied to a large variety of processes in addition to those which are thought of as ordinary chemical reactions. Among these are viscous flow, electrolytic conduction, diffusion, and others. The formulation can also be applied to complex reactions with the same ease as to simple reactions. The ease of application of the formal theory is, of course, not an unalloyed advantage, for in many cases care must be taken in interpreting its findings. The essential difference between Eyring's formulation

of rate theory and previous ones lies in the fact that Eyring stresses the *free energy* of activation rather than the heat or energy of activation. Eyring has also replaced the somewhat nebulous concept of "reactive" molecules, as differentiated from normal inactive ones, by the "activated complex." This complex is defined specifically enough so that its configuration and energy can be calculated if one has sufficient knowledge of the configurations of the normal reactant molecules together with the distribution of energy among their degrees of freedom.

Figure 1 gives a schematic representation of the manner in which the activated complex is related to the reactants and to the reaction products. In such a diagram the ordinate, in practically all cases, might be labeled either energy or free energy, though strictly speaking it is the free energy of activation which is the determining factor in reaction velocity. The reacting system, in order to pass

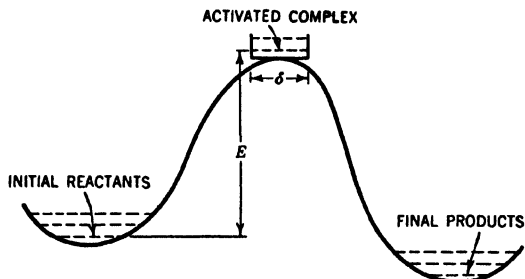


Fig. 1. Schematic representation of the mechanism of chemical reaction (54).

from the initial to the final state, must acquire the energy  $E$  enabling it to pass over the energy barrier. The system in the state represented by the top of the energy barrier is the activated complex. In the figure the curve gives the free energies of a series of reacting configurations between an initial condition of undistorted reactant molecules and undistorted reaction product molecules. The path followed by the curve represents the energies of the unique series of configurations which proceeds over the lowest possible maximum. This means that, except for one degree of freedom, the configuration at the top of the energy hill is stable with respect to all small displacements or distortions. Thus it has the properties of a stable

molecule except for one degree of freedom. This virtually unique configuration is, then, the "activated complex."

A striking result of statistical mechanics is that, regardless of the reaction involved, the specific rate of decomposition into reaction products, of those activated complexes formed from reactants, depends only on the temperature, and is equal to  $kT/h$ , where  $k$  is the Boltzmann constant (*i.e.*, the gas constant per molecule),  $h$  is Planck's constant, and  $T$  is the absolute temperature. Calculation of reaction velocity constants, then, can be made to depend only on a single assumption that there is a virtual equilibrium maintained between the activated complex and the reactants. On this assumption the rate formulation can be simply made and is thermodynamically satisfying. Thus, if  $K^\ddagger$  represents the equilibrium constant for the formulation of activated complex from reactant molecules, it will give directly the concentration of activated complex in equilibrium with unit concentrations of reactants (or in the general case with any "standard state" concentration of reactants). We would have then:

$$k' = \kappa(kT/h)K^\ddagger \quad (1)$$

where  $k'$  is the reaction velocity constant, and  $\kappa$  is a transition coefficient. It represents the probability that any activated complex will not return to the state of reactant molecules rather than proceed to that of the reaction products. For most ordinary reactions  $\kappa$  is close to unity, and will be so considered in this paper. Equation (1) neglects any quantum-mechanical "leakage" through the potential hill, which is negligible in reactions here considered.

As in any ordinary chemical reaction,  $K^\ddagger$  will be related to the changes in the ordinary thermodynamic magnitudes involved in activation. If  $\Delta F^\ddagger$ ,  $\Delta H^\ddagger$ , and  $\Delta S^\ddagger$  are the standard changes in free energy, heat content, and entropy, respectively, when the activated complex is formed from reactant molecules, we may write:

$$\Delta F^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger = -RT \ln K^\ddagger \quad (2)$$

and taking  $\kappa$  as unity we may rewrite equation (1):

$$k' = (kT/h) \exp(-\Delta F^\ddagger/RT) = \\ (kT/h) \exp(-\Delta H^\ddagger/RT) \exp(\Delta S^\ddagger/R) \quad (3)$$

For many purposes equation (3) is a convenient form for handling biological kinetic data.

Equation (3) gives the Arrhenius equation (2) if we take the logarithms of both sides and differentiate with respect to temperature. Thus, if  $\Delta S^\ddagger$  is virtually independent of temperature:

$$d \ln k'/dT = 1/T + \Delta H^\ddagger/RT^2 = (\Delta H^\ddagger + RT)/RT^2 = \mu/RT^2 \quad (4)$$

where we identify the Arrhenius  $\mu$  with  $\Delta H^\ddagger + RT$ . It is easy to see how the collision theory could have arisen by intuition from the Arrhenius equation. Integration of equation (4) yields  $k' = Ce^{-\mu/RT}$  (80), where  $C$ , embodying a frequency factor, is a measure of the probability that active molecules shall yield reaction products, and  $e^{-\mu/RT}$  is a Boltzmann factor interpreted as giving the fraction of molecules having excess energy  $\mu$  or more. An alternative interpretation of the Boltzmann factor, equally valid where it applies at all, is that it gives the ratio of the concentration of molecules having energy  $\mu$  in excess of that of the reference state to that of molecules having zero energy (*i.e.*, in the reference state). This latter, and equally valid, interpretation is just the equilibrium constant for the reaction:

$$M_0 = M_\mu \quad (5)$$

Thus we would write:

$$M_\mu/M_0 = K^\ddagger = e^{-\mu/RT} \quad (6)$$

The obvious inadequacy of this formulation is at least twofold. In the first place this form of Boltzmann factor applies only to energy changes affecting degrees of freedom involving a total of only two squared terms in their formulation. In the second place we use  $\mu$  for  $\Delta F^\ddagger$ , an energy change for a free energy change. Eyring's formulation removes both of these limitations. By use of the ratio of partition functions there is no formal limitation on the manner in which energy may distribute itself among *all possible* degrees of freedom; and, as pointed out above, the free energy of activation is stressed as the important factor.

In terms of partition functions equation (3) assumes the form:

$$k' = (kT/h) (Q^\ddagger/Q_r) \exp(-E_0/RT) \quad (7)$$

where  $Q^\ddagger$  and  $Q_r$  are, respectively, the partition functions of the activated complex, treated as a molecule, and of the reactant mole-



cules; and  $E_0$  is the excess energy of the ground state of the activated complex over that of the ground states of the reactant molecules. In his original formulation Eyring (22) pointed out explicitly the conditions under which the expression  $(kT/h)(Q^\ddagger/Q_r)$  assumes the form of expression usually employed in calculating the collision number,  $Z$ . He states: "If the two colliding molecules have (a) none of their internal frequencies appreciably modified in the activated state and (b) if the two degrees of freedom replacing translation, which are not themselves translation, correspond to a rotation (as in the very special case of two colliding atoms) or if they are bending frequencies with force constants of practically zero, then we are justified in applying the simple kinetic theory" which, for bimolecular reactions where activation results from collision, gives the rate:

$$k' = Ze^{-E/RT} \quad (8)$$

The negligibly small number of reactions which fit the above-mentioned specifications and for which equation (8) has been found to be satisfactory has led to the introduction of a so-called steric factor,  $P$ , so that:

$$k' = PZe^{-E/RT} \quad (9)$$

Equation (9) is obviously of universal applicability since  $P$  is virtually defined as  $k'/Ze^{-E/RT}$ . There is no theory for  $P$ . It has been called a steric factor and one can easily get an intuitive rationalization of cases in which  $P$  is less than unity. There are many cases in which  $P$  is much greater than unity (3) and in such cases it seems impossible to conceive why so many collisions which do *not* have the necessary energy should yet result in reaction. Stearn and Eyring (81) have treated this point analytically in some detail, as has Stearn in a former review of enzyme kinetics (80).

#### INFLUENCE OF PRESSURE

So long as reactions are studied at a single constant pressure, equation (3) is adequate for treating kinetic data from most biological processes studied. If processes are studied at different pressures it is convenient to have a formulation in which pressure appears explicitly. The pressure effect, which can be precisely formulated, can be made obvious in equation (3) by substituting for  $\Delta F^\ddagger$  its thermodynamic equivalent (24). Thus:

$$\Delta F^\ddagger = \Delta E^\ddagger + P\Delta V^\ddagger - T\Delta S^\ddagger \quad (10)$$

giving the rate equation:

$$k' = (kT/h)e^{-\Delta E^\ddagger/RT}e^{-P\Delta V^\ddagger/RT}e^{\Delta S^\ddagger/R} \quad (11)$$

In equation (11),  $\Delta V^\ddagger$  is the increase in volume when activation takes place, *i.e.*, it is the difference in volume between the activated complex and the reactants. Since, from equation (1):

$$-RT (d \ln k'/dP)_T = -RT (d \ln K^\ddagger/dP)_T = \Delta V^\ddagger \quad (12)$$

it is seen that the slope of the curve obtained by plotting  $RT \ln k'$  against pressure is  $-\Delta V^\ddagger$ . If  $RT$  is expressed in cubic centimeter atmospheres and the pressure in atmospheres,  $\Delta V^\ddagger$  will be obtained in cubic centimeters. The observed values of  $\Delta V^\ddagger$  provide an additional criterion by which one may study reaction mechanisms. For certain simple reactions Stearn and Eyring have been able to predict with marked success this volume change on activation from the structure of the reactants (82). Thus it is seen that a set of kinetic data involving measurements through both a temperature range and a pressure range will permit one to obtain values for  $\Delta E^\ddagger$ ,  $\Delta S^\ddagger$ , and  $\Delta V^\ddagger$ .

### III. Temperature Effects on Rates of Biological Processes

A study of the effect of temperature on the rates of processes yields values of  $\mu$  or of  $\Delta H^\ddagger$ . Data are more abundant covering this phase than covering pressure changes or giving absolute values of velocity constants in terms of definite standard states. This is true probably because one does not have to know purities of reagents and can thus work with crude preparations and yet obtain valuable data.

#### A. HEAT DENATURATION OF PROTEINS AND INACTIVATION OF ENZYMES

The heat inactivation of enzymes has come to be considered a particular case of protein denaturation, or of a process involving the same mechanism. The protein nature of enzymes and the similarity between the high values of  $\mu$  for the two processes has led to this association. Since these two processes are, in general, unimolecular, the problem of standard states does not enter and

we can from the temperature dependence, obtain both  $\Delta H^\ddagger$  and  $\Delta S^\ddagger$  values for activation.

TABLE I  
EXAMPLES OF PROTEIN DENATURATION AND ENZYME INACTIVATION

Protein or enzyme	Temp., °C.	$\Delta H^\ddagger^a$	$\Delta S^\ddagger^b$	$\Delta F^\ddagger^a$	pH	Ref.	Approx. P
Insulin	80	35,600	23.8	27,200	1.5	27	10 <sup>4</sup>
Trypsin	50	40,000	44.7	25,700	6.5	68	10 <sup>9</sup>
Emulsin	60	45,000	65.3	23,200	—	37	10 <sup>13</sup>
Solanain	60	61,300	109	25,100	7.0	34	10 <sup>20</sup>
Hemoglobin	60.5	75,600	153	24,700	5.7	58	10 <sup>22</sup>
Leucosin	55	84,300	185	23,620	6.1	20	10 <sup>26</sup>
Invertase (yeast)	50	110,400	263	25,500	4.0	20	10 <sup>56</sup>
Vibriolysin	50	128,000	326	22,700	—	26	10 <sup>70</sup>
Egg albumen	65	132,000	316	25,400	5.0	58	10 <sup>86</sup>
Tetanolysin	50	172,600	459	24,350	—	26	10 <sup>96</sup>
Hemolysin (goat)	50	198,000	537	24,600	—	26	10 <sup>115</sup>

<sup>a</sup> Cal. per mole.

<sup>b</sup> Cal. per mole per degree.

In Table I are gathered examples of protein denaturation and enzyme inactivation which show about the limits to be expected in values of  $\mu$  (or  $\Delta H^\ddagger$ , which is numerically nearly the same). All values in the table are rounded off. The large range through which values of  $\Delta H^\ddagger$  are found (the largest value nearly six times the smallest value) is to be contrasted with the approximate constancy of the  $\Delta F^\ddagger$  values, in which there is only 20% difference between the largest and the smallest values. Thus it is seen that in spite of the large difference in  $\mu$  values all these reactions take place at the same speed within a factor of about 10<sup>2</sup>. If we compare those studied at 50°C. we see that the difference in  $\mu$  values alone would correspond to a difference in rates of as much as 10<sup>107</sup> times in the most aggravated case. The experimental behavior is easily understandable, however, on the basis of the picture developed by Eyring and Stearn (25), when it is realized that denaturation is accompanied by an opening up of protein structure accompanied, even in the activated state, by an increase in entropy which may be very large.

Somewhat more extensive tables will be found in papers by Eyring and Stearn (25) and by Sizer (76). In Table I of Sizer (76) it should be pointed

out that not all values listed under the heading " $\mu$ " are energies of activation. The value 10,000, given for muscle catalase is the value reported for  $\mu/R$ . Possibly more confusing is the value given for luciferase. This value, 55,000 cal., coupled with the entropy change of 184 cal. per degree would give a *negative* free energy of activation at all temperatures above 27°C. These values are, in reality, for the equilibrium reaction, and not for the activation process. The same thing holds for the value 67,600 cal. given for trypsin. This is the actual heat of denaturation, the value of  $\mu$  being about 41,000 cal. In the present table, I have not included muscle catalase since the reaction by which it was inactivated was not unimolecular but involved oxygen.

Bull (7) concludes that all denaturation represents an unfolding of the native protein into an asymmetric polar molecule. Under optimum conditions for spreading, urea- and heat-denatured films are structurally similar to spread native films. Even in solution, he says, denatured proteins are unfolded asymmetric forms of the native protein molecule.

TABLE II

A. EQUILIBRIUM BETWEEN ACTIVE AND INACTIVE TRYPSIN*									
Temp., °K	Inactivation (meas.), %	Inactivation (Calc) <sup>b</sup> , %	K (meas.) <sup>c</sup>	$\Delta F$	$\Delta S$				
315.1	32.8	32.8	0.49	446	213.1				
317.1	50.0	50.0	1.00	0	213.2				
318.1	57.4	56.4	1.35	-190	213.1				
321.1	80.4	80.0	4.10	-901	213.3				
323.1	87.8	87.2	7.20	-1269	213.1				
B. ACTIVATION OF NATIVE AND DENATURED TRYPSIN AT 323°									
Denaturation					Reactivation				
$\Delta H^\ddagger$	$\Delta S^\ddagger$	$\Delta F^\ddagger$	P	n	$\Delta H^\ddagger$	$\Delta S^\ddagger$	$\Delta F^\ddagger$	P	n
40,200	44.7	25,700	10°	7	-27,400	-168.4	27,000	10 <sup>-40</sup>	10 <sup>-44</sup>

- \*  $\Delta F$ ,  $\Delta F^\ddagger$ ,  $\Delta H^\ddagger$  in cal. per mole;  
 $\Delta S$ ,  $\Delta S^\ddagger$  in cal. per mole per degree.  
<sup>b</sup> Using  $\Delta H = 67,600$  cal. per mole.  
<sup>c</sup> Inactive/active.

The large values of  $P$  met with in many cases can be explained only on the basis of large entropy increases on activation. These counterbalance the large activation energies and yield reasonable reaction rates where immeasurably slow ones might otherwise be expected. That Eyring's formulation does *not* constitute merely an alternative and essentially equivalent point of view to the collision

theory would be brought out even more clearly if we had data on the rates of the reverse of denaturation reactions. Many thermal denaturations, if not performed too rigorously, may be reversed. In the temperature range 40 to 50°, the heat denaturation of trypsin is readily and completely reversible, and Anson and Mirsky (1) have measured the equilibrium between active native trypsin and inactive denatured trypsin in this temperature range. In Table IIA are values obtained from their data. Part B of Table II makes use of Pace's data (68) on denaturation and the equilibrium data of Anson and Mirsky to obtain the values involved in the reactivation of trypsin. It is noted here that  $\Delta H^\ddagger$  is negative. This situation would be difficult to treat rationally on the basis of the collision theory. The value of  $P$  necessary to make equation (9) work is given for interest. The collision theory finds itself more at ease in handling bimolecular reactions than unimolecular ones. The formulation used, under proper conditions, for the latter class is:

$$k' = Be^{-E/RT} (E/RT)^n (1/n!) \quad (13)$$

Here  $B$  is a constant,  $E$  is related to the activation energy by:

$$E = \mu + nRT \quad (14)$$

and  $n$  is one less than the number of degrees of freedom involved in the activation process. It is seen in Table IIB that the use of equation (13) for the reaction there considered requires a value of  $n$  which has no physical significance.

In Figure 2 the heats, entropies, and free energies of activation for trypsin denaturation are compared to values for the equilibrium reaction.

The course of the denaturation is measured in terms of number of bridges broken. In each case the activated complex is represented by the point marked A. N denotes the native, and D the denatured, state of the protein. Unlike the majority of chemical reactions whose behavior is represented in Figure 1, the heat content does not pass through a maximum at the activated configuration. There is a progressive, almost linear, increase in heat content from native to completely denatured state. This reaction exemplifies the reason for the emphasis on free energy change in the general case. Here the entropy increases only slowly until the activated state is reached, but from there on it increases very rapidly. The result is that the free energy of the activated complex is a maximum, whereas its heat content and entropy are intermediate in value between the native and the denatured state.

The activated state is seen to be an intermediate configuration between the native and denatured state, and the process of acti-

vation in this case is essentially the same as the remainder of the process except for the relative values of entropy increase to heat content increase. During the activation-denaturation process the ratio  $T\Delta S^\ddagger/\Delta H^\ddagger$  is about 0.36, whereas that for the remainder of the process jumps to 2. A ratio of less than unity means that the process

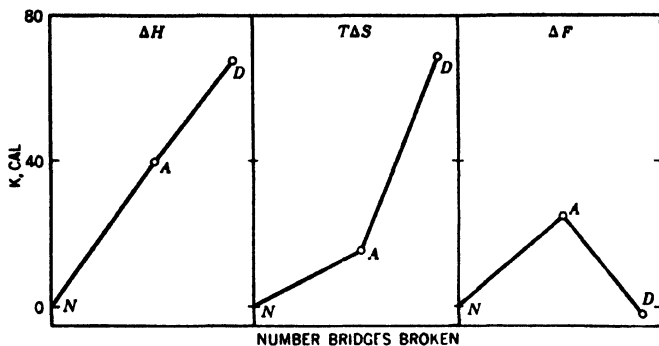


Fig. 2. Change in three thermodynamic quantities during denaturation of trypsin.

will take place only on the investment of free energy, whereas a ratio greater than unity denotes a spontaneous process in which free energy is liberated.

### B. MECHANISM

For reactions involving simple molecules it has been found, in general, in the activated molecule, that the lengths of bonds involved in reaction are increased by about 10% of their normal values; and the energy of activation is approximately one-fourth the sum of energies of the bonds involved (28). The high values of  $\mu$ , running well over 100,000 cal. in many cases, coupled with the fact that denaturation does not involve chemical decomposition in the sense of the splitting up of molecules, almost forces one to picture the mechanism of denaturation as the breaking of a large number of very weak bonds, such as hydrogen bridges. The breaking of a few strong bonds could never yield the enormous increases in entropy met with in many of these reactions. A picture of denaturation activation was developed by Eyring and Stearn (25) to account for all the facts. According to them the breaking of the

first few bonds will not greatly alter the configuration of the molecule and thus will yield only small increases in entropy. The difference between the energy necessary to break these bonds and the energy returned by entropy increase must be made up by an investment of free energy into the activation process. As Eyring and Stearn express it: "The breaking of these bonds will not pay their way." Ultimately when a sufficient number has been broken, the large molecule is no longer firmly held to its native configuration and the breaking of one or two additional bonds will result in

TABLE III<sup>a</sup>

Substance	$\Delta H^\ddagger$	$\Delta S^\ddagger(\text{mess.})$ (1)	Bridges broken, no. <sup>b</sup>	$\Delta S^\ddagger(\text{calc.})$ (2)	Ratio (1)/(2)
Enterokinase	42,200	52.8	8	96	0.55
Trypsin kinase	44,300	57.6	9	108	0.53
Proteinase, pancreatic	37,900	40.6	7	84	0.48
Lipase, pancreatic	45,400	68.2	9	108	0.63
Amylase, malt	41,600	52.3	8	96	0.55
Emulsin	44,900	65.3	9	108	0.60
<i>Average</i>					<i>0.56</i>
Pepsin	55,600	113.3	11	132	0.86
Leucosin	84,300	185.0	17	204	0.91
Egg albumen	132,000	315.7	26	312	1.01
Hemoglobin	75,600	152.7	15	180	0.85
Hemolysin, goat	198,000	537.0	40	480	1.12
Vibriolysin	128,000	326.0	26	312	1.04
Tetanolysin	172,600	459.0	36	432	1.06
Peroxidase, milk	185,300	466.0	37	444	1.05
Rennen	89,300	208.0	18	216	0.96
<i>Average</i>					<i>0.99</i>
Trypsin	40,200	44.7	8	96	0.47
Trypsin	67,600 <sup>c</sup>	213.0 <sup>d</sup>	14	168	1.27
Egg albumen					
pH 7.7	134,300	317.1	27	324	0.98
pH 3.4	96,800	223.7	19	228	0.98
Yeast invertase					
pH 5.7	52,400	84.7	10	120	0.71
pH 5.2	86,400	185.0	17	204	0.91
pH 4.0	110,400	262.5	22	264	0.995
pH 3.0	74,400	152.4	15	180	0.85
Egg albumen					
pH 1.35	35,200	36.3	—	—	—

<sup>a</sup>  $\Delta H^\ddagger$  in cal. per mole;  $\Delta S^\ddagger$  in cal. per mole per degree.

<sup>b</sup>  $\Delta H^\ddagger/5000$ . <sup>c</sup>  $\Delta H$ . <sup>d</sup>  $\Delta S$ .

complete collapse of structure with very large entropy increase. From such a state, the activated state, the reaction proceeds rapidly and spontaneously to completion. Thus we have seen that the activation entropy for trypsin is about 45 cal. per degree, whereas the total entropy increase is some 213 cal. per degree.

Stearn (80) studied this mechanism in a little more detail. He estimated that, if the general structure of the protein molecule is not affected by the breaking of a particular bond, the "local" entropy increase may be expected to be of the order of some 11–12 cal. per degree. Mirsky and Pauling (63) suggest about 5 kcal. as the strength of the NHO bond in proteins. Huggins (40) gives as "upper" limits for the strength of OHO 6.2 to 7.2 kcal., of CHN 6.7 to 8.4 kcal., of CHO 4.1 to 6.0 kcal., and NHO 6.0 to 6.8 kcal. The bonds broken will not all be the same, of course, but we shall take, arbitrarily, 5.0 kcal. for all such bonds involved. This would mean that in the activation of trypsin denaturation there would be  $\Delta H^\ddagger/5000 = 40,000/5000$ , or about 8 bonds involved, whereas in the complete reaction these would be 67,600/5000 or about 14 bonds of this nature broken. Table III was prepared from data such as those in Table I using 5 kcal. as the bond strength of the bonds broken and the value of 12 cal. per degree for the net gain in entropy per bond broken (25). These crude average values for bond strengths and entropy increases are not particularly good guesses in those cases in which the activation energies are low, but are better in those cases involving energies above 60,000 or so, where a dozen or more bonds are broken on activation and where averages begin to have significance. The agreement between the calculated and kinetically determined entropies of activation seems to be too consistently good to be purely fortuitous. It is evident that there are very few stronger bonds involved. This is directly supported by the fact that the process of denaturation renders detectable certain, or in some cases all, of the sulfhydryl, disulfide, and phenol groups, none or only a fraction of which are detectable in the native protein. Thus one of the steps in activation will frequently be the hydrolytic breaking of a cystine bridge which involves some 20,000 cal. and results in negligible entropy increase (25). When the total number of bridges broken is large this averages out fairly well, but when this number is small it results in a calculated entropy of activation significantly too large. Thus the average ratio of  $\Delta S^\ddagger$



(meas.)/ $\Delta S^\ddagger$  (calc.) for the first six examples in Table III, where the number of bridges broken seems to be less than 10, is only 0.56, whereas the average of this for the next nine examples is nearly unity. In fact had all fifteen examples been arranged in the order of increasing value of  $\Delta H^\ddagger$ , the ratio of measured to calculated entropy change would fall almost exactly in the same order.

Our specific knowledge, except in a few cases, does not justify any more detailed treatment than that given above, but the general picture indicates quite clearly that our average of 5000 per bond, which works well for large numbers of bonds, results from one or maybe 2 stronger bonds and several slightly weaker ones. As a matter of interest, if we calculate the number of bonds broken by the formula:

$$\frac{\Delta H^\ddagger - 20,000}{4000} + 1$$

*i.e.*, assuming 1 bond of 20,000 cal. yielding no entropy increase, and use 4000 cal. for the weaker bonds with 12 cal. per degree entropy change per "small" bond, the ratio in column 6 of Table III would average 0.91 for the first six examples in place of 0.56, whereas it would remain unchanged for the next nine examples, giving an over-all average for all fifteen of 0.95. The number of bonds broken, calculated in this way, is, in the first six examples, just 2 less than the number given in column 4 of Table III. In the other cases, if the number lies between 12 and 20, each method of calculation gives the same result, whereas above 20 this second method gives from 2 to 5 more than in column 4.

The figures for trypsin in Table III again illustrate the argument given above. During the activation process the bonds broken yield only about 5.5 cal. per degree of entropy, whereas after activation the remaining bonds when they break yield 28 cal. per degree per bond. At 50°C. it requires 15.5 cal. per degree for the process to just "pay its way," *i.e.*,  $T\Delta S^\ddagger$  would be just 5000 cal. to offset the  $\Delta H^\ddagger$  of 5000 cal. and give a  $\Delta F^\ddagger$  of 0. The large entropy yields after activation are not due to essential difference in bonds broken, but are due rather to the opening up of the molecule as a whole.

The data given in Table III for invertase and egg albumen at different pH values show that certain bonds, whether they are salt bridges or not, are apparently broken by ionization. For this class of bonds there will be naturally a pH of maximum stability, as is, indeed, generally observed, and is shown by the data for invertase. When such bonds are broken by ionization, the formulation of the activated molecule, of course, takes place from that state and thus

requires less energy of activation than when it starts from its most stable configuration. In fact it has been suggested (57,84) that the abnormally large entropies met with in these denaturation reactions are merely entropies of ionization of several acidic groups. However, even if *all* the 14 bonds apparently broken (Table III) when trypsin is denatured are of an ionic nature we could roughly assign  $213/14 = 15.2$  cal. per degree entropy increase to each bond broken. We compare this to the entropy of ionization of acetic acid under the same conditions (*i.e.*,  $pH = 2$ ,  $50^\circ$ ), roughly  $-12$  cal. per degree and we see at once that there must be another very important contribution to this quantity, and that these large entropy changes are quite real regardless of  $pH$  changes.

### C. BIOLOGICAL PROCESSES

The main difficulty in the study of biological processes lies in the large number of possible simultaneous reactions in such complicated systems. Occasionally one of such a possible group of reactions is preferentially catalyzed, so that, for practical purposes, its speed is the only one which need be considered. Thus one of the most important applications of kinetics is to enzyme-catalyzed reactions. In such cases we confine ourselves to two reactions, inactivation of enzyme and transformation of substrate. The temperature effect on reaction rate is frequently measured by the so-called temperature coefficient, symbolized by  $Q_{10}$ , giving the effect of a  $10^\circ$  temperature rise in reaction rate. Large values of  $\mu$  indicate large

TABLE IV\*

Enzyme	$Q_{10}$ , enzyme deactivation	$Q_{10}$ , catalysed substrate reaction	Substrate
Pancreatic lipase	15	1.2	Ethyl butyrate
Trypsin	8	1.8	Sturine
Emulsin	10	1.15	Salacin
Luciferase	12	2.3	Luciferin
Invertase	250 ( $pH$ 4)	1.5	Sucrose
Invertase	20 ( $pH$ 5.5)	1.5	Sucrose
Pepsin	16 ( $pH$ 6.4)	2.4	Casein
Amylase	7.8	1.9	Starch
Goat hemolysin	20,000	—	—

\* Data for these values were obtained in tables collected in references 76 and 80.

values of  $Q_{10}$ . For the thermal inactivation of enzymes the values of  $Q_{10}$  are frequently so large that these reactions change from slow to rapid in a very restricted temperature range, and thus enzymes may in general be treated as relatively stable at temperatures only slightly below a fairly specific "inactivation temperature." This is brought out in Table IV, where  $Q_{10}$  for the enzyme inactivation in its measured temperature range is compared to  $Q_{10}$  for a common substrate reaction with the same enzyme in its measured temperature range.

One cannot rest too securely, however, on this pictured reassurance. In Table V data are assembled from two independent studies, the amylase-catalyzed hydrolysis of starch and the thermal inactivation of amylase. The probable reason for the anomalous behavior of the starch-catalyzed reaction in this temperature range seems evident. The value of  $\mu$  for this reaction is the same at 25° as it is at 15°, and Sizer (76) gives this value as holding in the range 10–30°. At 35°, however, the value of  $\mu$  has decreased significantly (17,60) while at 45° it has diminished to about one-third its value at 25° (60).

TABLE V\*

Temp., °C.	Thermal inactivation of amylase, – log $k'$	– log $k'$	Starch hydrolysis by amylase: $\Delta H^\ddagger$ , cal./mole	$Q_{10}$	$k'/\text{starch reaction}$ $k'/\text{enzyme de-}$ activation
15	7.39	5.29	12,200	2.18	126.0
25	6.31	4.95	12,300	2.21	23.0
35	5.30	4.68	9,300	1.92	4.2
45	4.36	4.51	4,400	1.28	0.71

\* Data from references 17 and 60.

Up to 25° we are dealing with a reasonably simple reaction, since the inactivation of the enzyme is negligible compared to the starch hydrolysis. At 45°, however, the two rates are essentially equal, that of the enzyme inactivation being apparently slightly greater. Had the above experiments been extended to slightly higher temperatures we would probably have been faced with the seemingly absurd condition that  $\Delta H^\ddagger$  becomes negative and later becomes positive again when the rate of the uncatalyzed reaction becomes dominant. It is probably behavior of this nature which has led many to the conclusion that enzyme-catalyzed reactions do not

follow the Arrhenius equation (19,65). When this problem is studied more specifically, however, it is found that as long as we are dealing with the *same* reaction the Arrhenius equation will describe the temperature effect on the rate (32,73). Chase and Lorenz (11) noted an analogous situation in the oxidation of luciferin in the presence of luciferase. There are two simultaneous reactions, one luminous and the other nonluminous. In the temperature range 10–35° the over-all reaction is first order but the non-luminous reaction increases uniformly with temperature with a  $\mu$  of about 25,000 cal. In the low temperature range the luminous reaction accounts for about 95% of the total but it increases slowly with temperature ( $\mu = 7000$  cal.) and at about 22°  $\mu$  becomes –50,000 cal. For the over-all reaction  $\mu$  is about 6500 cal. up to about 25° and then becomes negative (–8600) through the remainder of the temperature range.

Claus (12) has studied the kinetics of the  $\beta$ -amylase action in 20% starch pastes from 10–69°. He finds that  $Q_{10}$  for the starch hydrolysis becomes unity at about 65° and less than unity in the range studied above this temperature. The frequently reported optimum temperature behavior of enzyme-catalyzed reactions represents the effect of temperature on enzyme inactivation in competition with the effect on the catalyzed reaction.

#### D. CHANGE OF $\mu$ WITH TEMPERATURE

Sizer and Josephson (78) have noted an interesting change in the value of  $\mu$  for enzyme catalyzed reactions which seems to occur within a very small temperature interval. They studied three cases: inversion of sucrose by invertase, hydrolysis of casein by trypsin, and hydrolysis of tributyrin by lipase. They state that no adequate explanation has been offered, but suggest that the transition may be due to a shift in the enzyme molecule "from one configuration to another" (76). Sizer has also noted a slight shift in  $\mu$  in the urease decomposition of urea which occurs at a definite temperature when the system is poised at an intermediate oxidation-reduction potential. Figures 3 and 4 show the two types of changes.

In Figure 4 it is seen that in a mildly oxidizing environment  $\mu$  is constant over the entire temperature range and has the value 11,700 cal. (curve 1). In a mildly reducing environment the value of  $\mu$  is 8700 cal. over the entire temperature range (curve 3). For intermediary oxidation-reduction potentials

$\mu$  at low temperatures has the value 11,700 cal. characteristic of the oxidizing environment, but at 22° there is a break in the curve and above this temperature  $\mu$  has the value 8700 cal. characteristic of the reducing environment.

It is seen that the change in oxidation-reduction potential induces a relatively small change in  $\mu$  and the effect may well be due to

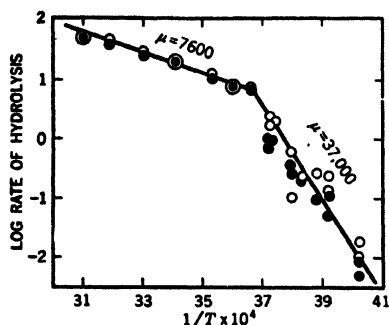


Fig. 3. Sharp break in value of  $\mu$  for tributyrin hydrolysis by pancreatic lipase at 0° C. (78).

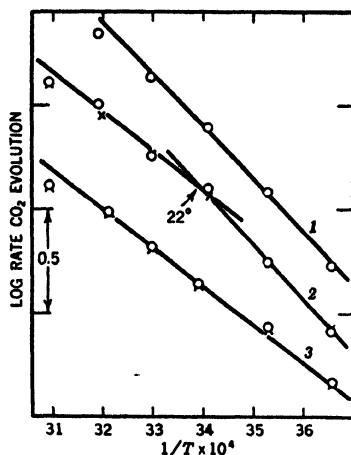
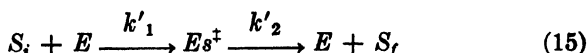


Fig. 4. Effect of environment on behavior of  $\mu$  in jack bean urease-urea system (74).

some modification of the enzyme molecule. However, it seems to this writer that the temperature effect in the other cases is rather large to be accounted for in this way. The values obtained were:

System	$\mu$ , cal.	
	Above transition temp.	Below transition temp.
Urea-urease	8,700	11,700
Tributyrin-lipase	7,600	37,000
Casein-trypsin	15,400	65,000
Sucrose-invertase	11,100	60,000

It is rather suggested that two consecutive reactions are involved in all cases. They can be crudely represented by the equation:



where  $E$  denotes enzyme,  $S_i$  denotes the initial substrate, and  $S_f$  the final product. The reaction indicated by  $k'_1$  would probably proceed with an entropy decrease whereas that denoted by  $k'_2$  may well show an entropy increase. We express the  $k'$  values conventionally:

$$k'_1 = P_1 Z_1 e^{-\mu_1/RT}$$

$$k'_2 = P_2 Z_2 e^{-\mu_2/RT}$$

and define  $P'$  so that  $P' = P_2 Z_2 / P_1 Z_1$ . Then:

$$k'_2/k'_1 = P' e^{-(\mu_2 - \mu_1)/RT} \quad (16)$$

Table VI shows the observed behavior of such systems.

We note that at low temperatures  $k'_2$  is much less than  $k'_1$  and is thus the rate-determining step, whereas at higher temperatures this step overtakes that represented by  $k'_1$  and the latter becomes the rate-determining step. We evaluate  $P'$  by setting the rates equal at the critical temperature value and then note that 5° from this temperature in either direction the rates differ by a factor of 3 to 5 or more except for the urease system. It would be of interest to know how general this type of behavior is and whether the temperature at which the shift in  $\mu$  is observed is nearly the same in a number of cases as it seems to be in the cases studied. It happens that this shift takes place at the temperature at which dilute aqueous solutions freeze, but freezing does not seem to be the cause of the change since solutions containing glycerol which froze in the neighborhood of -18° were studied and the change in  $\mu$  still was noted at 0°.

It is impossible from data in these cases to calculate any standard entropies of activation since we do not know the concentrations of all the reactants, particularly the enzymes. However, the same stock solutions of reagents were used throughout so that *comparative* values of the entropy of activation above and below the temperature of shift in  $\mu$  will have significance. In Table VII values of  $\Delta S^\ddagger$  are from unimolecular reaction velocity constants, estimated from Sizer's data. It should be emphasized that the significance of these values depends less on the method of obtaining them than on uniformity, since they are of comparative interest only. In the

TABLE VI

CHANGE IN  $\mu$  AT A CRITICAL TEMPERATURE

Sucrose-invertase			Casein-trypsin			Tributyrin-lipase			Urea-urease		
Temp., °C.	$\frac{-(\mu_2 - \mu_1)}{RT}$	$\frac{k'_2}{k'_1}$	Temp., °C.	$\frac{-(\mu_2 - \mu_1)}{RT}$	$\frac{k'_2}{k'_1}$	Temp., °C.	$\frac{-(\mu_2 - \mu_1)}{RT}$	$\frac{k'_2}{k'_1}$	Temp., °C.	$\frac{-(\mu_2 - \mu_1)}{RT}$	$\frac{k'_2}{k'_1}$
20	$10^{-24.5}$	450	20	$10^{-27.0}$	510.0	20	$10^{-21.94}$	40.0	50	$10^{-2.90}$	1.56
10	$10^{-27.76}$	25	10	$10^{-28.20}$	26.0	10	$10^{-22.70}$	7	40	$10^{-2.08}$	1.35
5	$10^{-28.44}$	5	5	$10^{-28.92}$	6.0	5	$10^{-22.98}$	2.9	27	$10^{-2.108}$	1.09
0.2	$10^{-28.15}$	1	0.2	$10^{-28.71}$	1.0	0.2	$10^{-22.54}$	1.0	22	$10^{-2.222}$	1.0
-4.5	$10^{-28.80}$	0.18	-4.5	$10^{-29.46}$	0.18	-4.5	$10^{-22.88}$	0.36	17	$10^{-2.281}$	0.92
-10	$10^{-29.65}$	0.03	-10	$10^{-31.22}$	0.03	-10	$10^{-24.44}$	0.105	7	$10^{-2.212}$	0.76
-15	$10^{-31.44}$	0.005	-15	$10^{-32.02}$	0.0048	-15	$10^{-24.92}$	0.04	0	$10^{-2.202}$	0.66
$\mu_2 = 60,000$ cal.			$\mu_2 = 65,000$ cal.			$\mu_2 = 37,000$ cal.			$\mu_2 = 11,700$ cal.		
$\mu_1 = 11,100$ cal.			$\mu_1 = 15,400$ cal.			$\mu_1 = 7600$ cal.			$\mu_1 = 8700$ cal.		
$P' = 10^{28.15}$			$P' = 10^{28.71}$			$P' = 10^{22.54}$			$P' = 10^{2.222}$		

TABLE VII  
COMPARATIVE ACTIVATION ENTROPIES FOR SYSTEMS IN WHICH  $\mu$  ALTERS WITH TEMPERATURE\*

Tributyryl-lipase		Sucrose-invertase		Casein-trypsin		Urea-urease	
Temp., °C.	$\Delta S^\ddagger$ (no glycerol)	Temp., °C.	$\Delta S^\ddagger$ (glycerol)	Temp., °C.	$\Delta S^\ddagger$	Temp., °C.	$\Delta S^\ddagger$
50	-54.8	50	-43.0	—	—	—	—
40	-54.4	40	-41.8	—	—	—	—
30	-54.4	30	-41.7	30	-29.1	50	-57.5
20	-54.0	20	-41.7	20	-28.8	40	-57.5
10	-54.4	10	-41.5	10	-29.3	27	-58.4
5	-54.4	—	—	—	—	—	—
0.2	—	0.2	—	0.2	—	22	—
-4.5	+54.0	-4.5	+139.0	-4.5	+152	17	-46.1
-10	+52.3	-10	+144.0	-10.0	+148	7	-45.6
-15	+53.2	-15	+140.0	-15	+150.5	0	-44.9
-18	+54.7	-18	+139.0	—	—	—	—
-24.5	+53.6	—	—	—	—	—	—

\*  $\Delta S^\ddagger$  in cal. per degree.



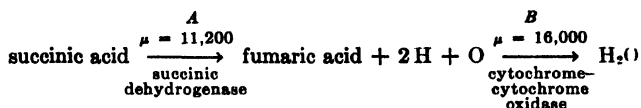
case of the urea-urease reaction there is little change in entropy of activation and no apparent change in mechanism.

With the other systems, however, the activation entropy remains constant over a temperature range in some cases as great as 40 to 45°C., and then, in a range of less than 10°, changes to a new value, which, in turn remains constant in the range studied, in some cases 20 to 25°. This suggests that at low temperatures the slow or rate-determining step is one of large  $\mu$  and large  $\Delta S^\ddagger$ . These conditions would be met by the reaction of constant  $k'_2$  (equation 15) in which the heat of breaking the bonds holding enzyme and substrate together will tend to increase the value of  $\mu$ . At the same time this dissociation may well involve an entropy increase if there is not too great an increase in the polarity of the dissociation products over the activated complex. On the other hand the reaction of constant  $k'_1$  may be expected to have a lower value of  $\mu$  since the formation of the enzyme-substrate bonds will liberate some of the energy necessary to activate the substrate molecule. In addition, the tying up of substrate molecules by enzyme may well result in an entropy decrease. If the above suggestion is found to be the explanation of the change in  $\mu$  due to change in temperature, it may be suggested that, in those cases in which the low temperature, slow step does not overtake the other step until heat inactivation of the enzyme takes place, no such phenomenon will be observed. More work is necessary to clear up this point.

This same sudden shift in  $\mu$  at a certain temperature has also been reported more recently by Bernheimer (4), who determined temperature coefficients of rates of hemolysis by various lipids. As Sizer had previously noted, Bernheimer found that in the instances in which shifts in  $\mu$  were encountered there was a low temperature range with a large value of  $\mu$  prevailing and, above a certain temperature, another range with a lower value of  $\mu$  prevailing. Thus, for tetanolysin,  $\mu$  between 0 and 15° was 40,000 cal., and between 15 and 30°, was 21,200 cal.; for streptolysin O,  $\mu$  from 0 to 10° was 43,000 cal. and, from 20 to 30°, was 21,400 cal.

Besides temperature and oxidation-reduction potential, it has been found that in certain cases specific reagents may alter the value of  $\mu$  without changing the order of magnitude of the reaction velocity. For example, Hadidian and Hoagland (35) found that addition of a small amount of cyanide does not alter the  $\mu$  value in

the case of oxygen consumption by the succinate-succinic dehydrogenase-cytochrome-cytochrome oxidase system of beef heart. The addition of slightly larger amounts causes an increase in the  $\mu$  value from 11,200 to 16,000 cal. This change is due to a sequence of two successive reactions as follows:



For the normal unpoisoned system, reaction *A* is the slow step and determines, in the temperature range studied, the measured value of  $\mu$  for the over-all process. Sodium cyanide poisons the oxidase system selectively and progressively. When a small amount of cyanide is added the measured  $\mu$  is not changed, but when enough cyanide is added to render *B* the slow reaction, it becomes the pacemaker and the measured value of  $\mu$  alters from 11,200 to 16,000 cal. Subsequent addition of selenite, which selectively poisons the dehydrogenase, will bring back the value of  $\mu$  to 11,200 cal. for the over-all process.

On the whole, however, the values for particular enzyme-substrate systems seem independent of many environmental changes which may appreciably affect the reaction velocity. The effects of these factors are largely reflected in changes in activation entropy. Sizer (72) points out that, in the case of yeast invertase, the activation energy is independent of *pH* at least in the range from about 3 to about 8; it does not vary with ionic strength nor with changes in concentration of either enzyme or substrate. This would obviously mean that purity of enzyme will not affect  $\mu$  unless the impurities are specific poisons (75,77,90). It might seem that activation energies should be independent of the particular substrate, since the type of substrate linkage attacked by a particular enzyme may well be the same in different classes of substrates. Actually this is frequently found to be the case, but by no means universally, and unless the enzyme-substrate bonding involves nearly the same energy for different substrates it is not surprising that activation energies sometimes vary from substrate to substrate for the same enzyme (9,31,35,36,56,77).

The idea that a particular  $\mu$  value can be associated with a

definite enzyme system has led Crozier to the idea that the determination of  $\mu$  for a physiological process should permit one to identify the enzyme system determining the over-all rate (13). This is rendered plausible by the observation that such processes follow the Arrhenius equation over a biologically large temperature range or until some critical temperature is reached, in which case the reaction shows one  $\mu$  value below this temperature and another one above. It is also noted that the  $\mu$  values are not large in number but tend to be grouped about a few modes. Crozier lists in one

TABLE VIII

Action	$\mu$ , cal.	Examples
Nerve center discharge	12,200	Creeping of ants Chirping of crickets Flashing of fireflies Neurogenic heart beat (ganglion alone subjected to temperature change)
Dehydrogenase oxidation	16,700	Phototropic orientation of <i>Limax</i> $O_2$ utilization in many species Respiratory rhythms in many insects Respiratory rhythms in goldfish Heart beat frequency in <i>Limax</i>
Hydroxyl-ion-catalyzed oxidation	11,500	Certain heart beat frequencies, $O_2$ utilization, etc.
Iron-catalyzed oxidation	16,200	Respiration in sea urchin eggs, etc.

group some fifteen biological processes, including cardiac, intestinal, and respiratory movements as well as polypregeneration, which show values of  $\mu$  similar to the value for oxidative reactions. He remarks: "It is difficult not to believe that these various processes ... involve some common fundamental process of a (relatively) simple character." It is suggested that, in a sequential series of catalyzed reactions which would be involved in a physiological process, the slowest of the series is the pacemaker or master reaction and determines the  $\mu$  value for the over-all rate. A shift in the

$\mu$  value is interpreted as indicating that a new step in the series has become the pacemaker. In Table VIII is a number of examples of processes which show essentially the same  $\mu$  value. These are collected from a series of papers by Crozier (13,14). The criticisms of Crozier's suggestion do not involve its empirical basis. They rather involve the identification of the  $\mu$  value for such a process with that of any simple catalyzed reaction. Crozier's idea has been criticized by Booij and Wolverkamp (5), and, implicitly, by the ideas presented in a paper by Burton (8). These authors suggest that, to understand *in vivo* processes kinetically, formulation should be made for steady states rather than for those ordinarily encountered *in vitro*.

#### IV. Kinetics of the Steady State

It seems worth while to inquire a little more minutely into the suggestion of Crozier by formulating steady state behavior in slightly more detail than is done in the suggestive paper of Burton (8). Two possibilities suggest themselves. There may be, in a particular physiological process, a series of successive reaction steps leading from the initial to the final state. This can lead to changes in the  $\mu$  values for the over-all rate when the temperature range is altered or when other environmental conditions are changed. In the second place, there may be simple reactions which could, conceivably, be catalyzed by more than one catalyst, so that there are alternate simultaneous mechanisms for the process. Such a process will also in general lead to changes in  $\mu$  values as the temperature range is altered or as changing environment causes one or the other mechanism to take over. In the second case it will be the fast reaction which would act as pacemaker. We shall consider the two possibilities in the order named.

##### A. SUCCESSIVE REACTIONS

Consider a series of reaction steps such that the initial reactant is maintained at a constant steady state concentration by some such process as diffusion or dissociation from a source of large supply, denoted by S, and the final product is steadily removed, by precipitation or diffusion or equivalent process, to a sink reservoir, denoted by R. For simplicity we shall assume that all reactions



In the present case we lose no generality by setting  $[S] = 1$  and  $[R] = 0$ , in which case equation (20) takes the form:

$$V = \frac{k_0 k_1 k_2 k_3 k_r K_1 K_2 K_3}{k_1 k_2 k_3 k_r K_1 K_2 K_3 + k_0 k_2 k_3 k_r K_1 K_2 K_3 + k_0 k_1 k_3 k_r K_2 K_3 + k_0 k_1 k_2 k_r K_3 + k_0 k_1 k_2 k_3} \\ = \frac{\prod k_i \prod K_i}{\alpha + \beta + \gamma + \delta + \epsilon} \quad (21)$$

If, then,  $\mu$  represents an activation energy for the over-all rate calculated by the Arrhenius relation:

$$\mu_V = RT^2 (d \ln V / dt)$$

or: 
$$\log \frac{V_2}{V_1} = \frac{\mu}{4.575} \left( \frac{1}{T_1} - \frac{1}{T_2} \right)$$

its value may or may not be close to any individual  $\mu$  characteristic of a certain step, and the relationships are not easy to determine in general. Thus:

$$\ln V = \sum \ln k_i + \sum \ln K_i - \ln (\alpha + \beta + \gamma + \delta + \epsilon) \quad (22a)$$

$$\mu_V = \sum \mu_i + \sum \Delta H_i - f(\mu_i, \Delta H_i) \quad (22b)$$

If any single term in the denominator of the fraction in equation (21) predominates, the problem is simplified. For example, if, in a certain temperature range,  $\beta$ ,  $\gamma$ ,  $\delta$ , and  $\epsilon$  can all be neglected compared to  $\alpha$ :

$$V = k_0 \quad \text{and} \quad \mu_V = \mu_0$$

Table IX indicates the relations between  $\mu_V$  and the values of  $\mu_i$ ; and  $\Delta H_i$ , for analogous simplified conditions. In a sufficiently wide temperature range  $\mu_V$  may actually change sign.

TABLE IX

Dominant value	V	$\mu_V$
$\alpha$	$k_0$	$\mu_0$
$\beta$	$k_1$	$\mu_1$
$\gamma$	$k_2 K_1$	$\mu_0 + \Delta H_1$
$\delta$	$k_3 K_1 K_2$	$\mu_0 + \Delta H_1 + \Delta H_2$
$\epsilon$	$k_r K_1 K_2 K_3$	$\mu_r + \Delta H_1 + \Delta H_2 + \Delta H_3$

The behavior of a system such as treated above is shown in Figures 5 and 6, where  $-\log V$ , calculated from equation (21) is plotted against  $1/T$ . The numerical data arbitrarily assumed for  $280^\circ\text{K}$ . were:

Value	Fig. 5	Fig. 6
$k_0$	$10^{-4}$	$10^{-4}$
$\mu_0$	4,000	4,000
$k_1$	$10^{-4}$	$10^{-4}$
$\mu_1$	8,000	6,000
$k_2$	$3 \times 10^{-4}$	$10^{-4}$
$\mu_2$	12,000	12,000
$k_3$	$10^{-4}$	$10^{-4}$
$\mu_3$	18,000	18,000
$K_1$	3.33	10
$\Delta H_1$	1,000	-6,000
$K_2$	3.00	10.0
$\Delta H_2$	1,000	-6,000
$K_3$	2.5	10.0
$\Delta H_3$	1,000	-6,000

The temperature range covered is, of course, wider than that through which enzymes can retain their activity but the same behavior would be noted in other ranges with different values of constants. Two things should be noted in Figure 5; there are ranges through which the curve is nearly a straight line and yet there are comparatively sudden changes in slope. The points have been connected by a few possibly somewhat arbitrary straight lines.

If we consider segments *B* and *C* of Figure 5, the slopes correspond, respectively, to  $\mu$  values of about 7800 and 11,400 cal. These are not too far from the  $\mu_1$  value of 8000 and  $\mu_2$  value of 12,000 cal. But even this rough agreement is somewhat fortuitous. In region *B* the values of  $\alpha$ ,  $\beta$ , and  $\gamma$  are of the same order and large compared to  $\delta$  and  $\epsilon$ , whereas in region *C*,  $\beta$  and  $\delta$  are the two terms in the denominator of equation (21) which need be considered. In region *D* and specifically in region *E*,  $\delta$  predominates. Here  $\mu_V$  should be  $\mu_3 + \Delta H_1 + \Delta H_2$ , or 18,000 cal., which is precisely the value obtained from the slope of segment *E*.

The data postulated for the reaction chain whose behavior is shown in Figure 6 yield results such that through most of the temperature range investigated  $\beta$  is the dominant term. This curve,

except in the high temperature range, yields essentially a single  $\mu_V$  value of about 6100 cal., quite close to the  $\mu_1$  value of 6000 cal. taken for one of the steps. However, in the high temperature range  $\epsilon$  takes over.

According to Table IX this should give a  $\mu_V$  of  $\mu_r + \Sigma \Delta H_i = -14,000$  cal. The slope of the segment joining the last two points

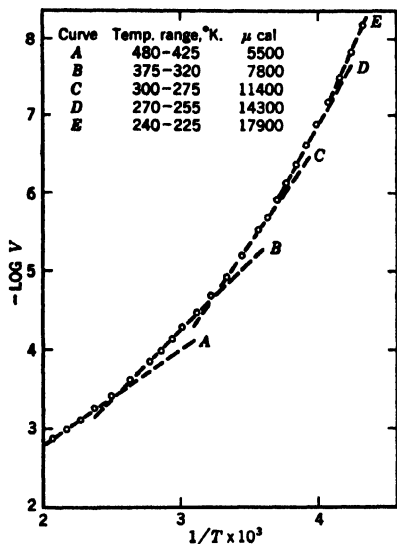


Fig. 5. Negative logarithm of over-all steady state velocity plotted against reciprocal of temperature, showing the effect of temperature on  $\mu$  for a specific set of sequential reactions. This behavior is specific for a particular process. See Fig. 6 for an analogous but different process.

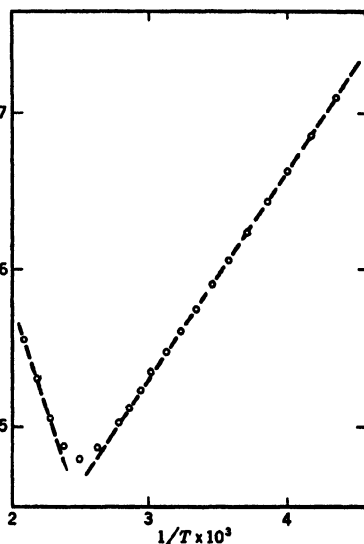


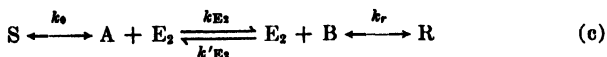
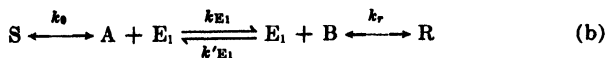
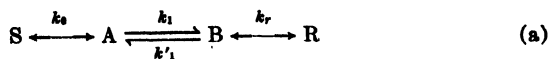
Fig. 6. Negative logarithm of over-all steady state velocity plotted against reciprocal of temperature, showing that there may be an actual change in sign of apparent  $\mu$  in a sequential series of processes in steady state at a certain temperature.

yields actually  $-13,500$  cal. In this case  $\mu$  for the over-all reaction has actually changed sign whereas those for the various steps have been assumed constant throughout the entire temperature range.

### B. SIMULTANEOUS REACTIONS

Next consider the process which can take place by way of three simultaneous paths, namely:





where  $E_1$  and  $E_2$  are different catalysts. We assume that the catalysts are effective over the entire temperature range investigated.

As before we can calculate rates for each path  $V_1$ ,  $V_2$ , and  $V_3$ , and the over-all rate of the process will be the sum of the rates over the individual paths. Treating the system as we did the sequential reaction series we find for these rates, putting  $S = 1$  and  $R = 0$ :

$$V_1 = \frac{k_0 k_1 k_r K}{k_1 k_r K + k_0 k_r K + k_0 k_1} = \frac{K \prod k_{1i}}{\alpha_1 + \beta_1 + \gamma_1} \quad (23a)$$

$$V_2 = \frac{k_0 k_{E_1} k_r K E_1}{k_{E_1} k_r K E_1 + k_0 k_r K + k_0 k_{E_1} E_1} = \frac{E_1 K \prod k_{2i}}{\alpha_2 + \beta_2 + \gamma_2} \quad (23b)$$

$$V_3 = \frac{k_0 k_{E_2} k_r K E_2}{k_{E_2} k_r K E_2 + k_0 k_r K + k_0 k_{E_2} E_2} = \frac{E_2 K \prod k_{3i}}{\alpha_3 + \beta_3 + \gamma_3} \quad (23c)$$

Obviously the equilibrium constant will be independent of the presence or absence or type of catalyst. For purposes of studying the behavior represented in Figures 7 and 8, we specify the following data and conditions for 280°K.:

TABLE X

Value	No catalyst	Catalyst 1	Catalyst 2
$k_0$	$10^{-4}$	$10^{-4}$	$10^{-4}$
$\mu_0$	4,000	4,000	4,000
$k$	$7.39 \times 10^{-11}$	$10^{-4}$	$9.5 \times 10^{-5}$
$\mu$	30,000	18,000	12,000
$k'$	$7.39 \times 10^{-12}$	$10^{-6}$	$9.5 \times 10^{-7}$
$\mu_1$	35,000	23,000	17,000
$k_r$	$10^{-4}$	$10^{-4}$	$10^{-4}$
$\mu_r$	4,000	4,000	4,000

Thus  $K$  at 280°K. is 100;  $\Delta H$  is -5000 cal. In all cases the catalyst concentration is arbitrarily set at  $10^{-5}$ .

In Figure 7 the logarithms of individual rates are plotted against temperature. In the low temperature range the master path is that catalyzed by  $E_2$ , in this case the fast path. At  $280^\circ\text{K}$ . the path catalyzed by  $E_1$  has the same rate and above  $280^\circ\text{K}$ . this path takes over and becomes the master path. At about  $320^\circ\text{K}$ . the uncatalyzed reaction with its higher temperature coefficient becomes the fast

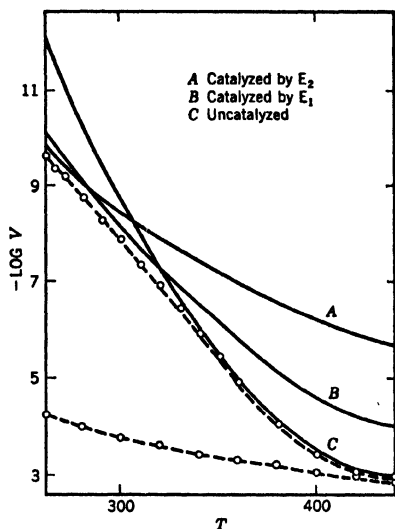


Fig. 7. Negative logarithm of reaction rate plotted against temperature for the steady state process taking place over three concurrent paths. (A) reacting system catalyzed by enzyme  $E_2$ ; (B) reacting system catalyzed by enzyme  $E_1$ ; (C) reacting system uncatalyzed.

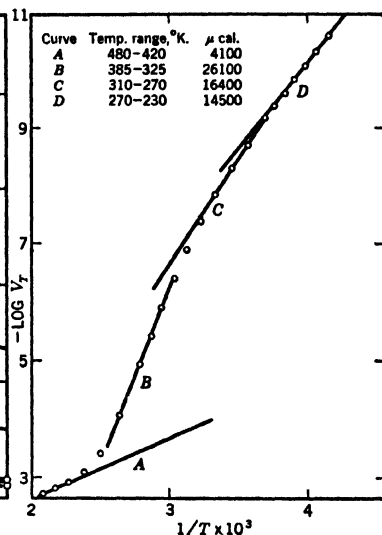


Fig. 8. Negative logarithm of total steady state velocity of a system that can undergo the same reaction over three concurrent paths plotted against reciprocal of the temperature, showing behavior of apparent  $\mu$  over a temperature range.

step. The over-all rate, shown by the upper broken line, is finally slowed up by the limiting process represented by  $k_0$ . The lower broken line represents what the over-all velocity would be if diffusion were the limiting process.

The lower broken line giving the values of  $\log k_0$  finally becomes identical with the over-all rate. This over-all rate seems to wobble about that of the master path or fast path. It approaches quite close

where one path is greatly predominant and deviates by a minimum of  $\log 2$  where two curves intersect, *i.e.*, where the over-all rate is at least twice that of either of the faster paths.

In Figure 8 we give the conventional plot of  $\log V$  against  $1/T$ . In this case the  $\mu_V$  values cannot be predicted as easily as in the case of the catenary type of reaction. Even if a particular path largely determines the over-all rate, as for example that catalyzed by  $E_2$  in the temperature range 240 to 250°K., the value of  $\mu_V$  will depend on the relative magnitudes of  $\alpha_1$ ,  $\beta_1$ , and  $\gamma_1$  in equation (23a). It turns out that  $\beta_1$  is large compared to  $\alpha_1$  and  $\gamma_1$ , so that if this were the only path we would expect a  $\mu_V$  of 12,000 cal. Reference to Figure 8, however, indicates that the rate by the path catalyzed by  $E_1$  is rapidly overtaking that of the path catalyzed by  $E_2$  and the over-all rate is increasing slightly faster with temperature than is that of the dominant single path. The slope of the curve of Figure 8 in this region gives 12,600 for  $\mu_V$ . In the region where path 2 is dominant one obtains  $\mu_V = 15,900$  compared to 18,000 for  $\mu_{E_1}$ . Soon after the uncatalyzed reaction becomes dominant but before  $k_0$  becomes the limiting factor,  $\mu_V$  reaches a value of about 28,000 cal. This compares with 30,000 for  $\mu$  for the uncatalyzed reaction. At higher temperatures, as the over-all rate approaches  $k_0$ ,  $\mu_V$  drops and would eventually reach  $\mu_0$ . In the range here noted it has reached the value 4600 as compared to 4000 for  $\mu_0$ .

By studying the behavior of these two classes of systems over a temperature range wider than any likely to be encountered in practice the general picture emerges more clearly than if the temperature range were restricted. Had one measured (or calculated) a few rates in a small range and met with values of  $\mu_V$  reasonably close to those for an individual path one would have been tempted to associate any measured value of  $\mu$  with that for a step which was playing the role of master process. This, it is seen, is an assumption which is frequently far from true though in limiting conditions it may be nearly so. Otherwise, for data, unless they be especially chosen, any agreement between  $\mu_V$  and a particular  $\mu$  for an elementary step is fortuitous.

## V. Affinity Constants of Enzyme-Substrate Complex

With the building of the enzyme into the activated complex in homogeneous systems it is natural to expect some type of associa-

tion between enzyme and substrate molecule. The slow step in the over-all reaction may be the formation of this association compound or it may be confined to a distortion of the substrate molecule or molecules which may accompany or follow the formation of the association compound. For reactions not otherwise complicated this would lead to kinetic results such as those reported by Hopkins (39) from a study of the hydrolysis of starch by amylase. If the substrate is present in excess the reaction is zero order with respect to substrate and first order with respect to enzyme; if the enzyme is present in excess the reaction is first order with respect to substrate. In this paper Hopkins gives the affinity constants of  $\alpha$ - and  $\beta$ -amylase for starch, expressed as per cent, as 0.024 and 0.3, respectively, and states that, therefore, the  $\alpha$ -amylase acts 0.3/0.024 or about twelve times as fast on starch as does the  $\beta$ -amylase. This would suggest that in these reactions the slow step in activation is not the formation of the enzyme-substrate compound. Van Slyke (88) emphasizes the generality of the behavior in which the apparent order of substrate reaction passes from zero order at high substrate concentrations to first order at sufficiently low substrate concentrations.

Formation of enzyme-substrate association compounds is further suggested by the tendency of substrate to protect the enzyme to a certain extent from inactivation by heat or other agents. The smaller the value of the Michaelis-Menten constant,  $K_m$ , which is the dissociation constant of the enzyme-substrate complex, the greater the protection of enzyme by substrate (16). Very little work has been done on the thermodynamics of this complex formation. The value of  $K_m$  increases with temperature in the few cases studied, and varies with pH. Van Slyke emphasizes the effect of pH on  $K_m$  (88), and Greenberg and Mohamed (33) studied this effect on  $K_m$  for the system arginine-arginase. In this case they define the "true" constant as the minimum value. It is related to the experimental value by the relation:

$$K_m (\text{true}) = K_m (\text{exp}) / \left( \frac{K_1}{c_{H^+}} + 1 \right) \left( \frac{c_{H^+}}{K_2} + 1 \right)$$

where  $K_1 = (H^+)(A^-)/(A^+)$ , the ionization constant of arginine, and  $K_2 = (H^+)(\text{enzyme}^-)/(\text{enzyme}^-)$ , the ionization constant of the enzyme.

Direct spectroscopic demonstration of enzyme-substrate compounds has been reported in the cases of the systems catalase-ethyl hydrogen peroxide (85) and peroxidase-hydrogen peroxide (51). The stability of these compounds as measured by the values of  $K_m$  vary over a wide range from such high values as about unity or greater (21,33,91) to  $10^{-7}$  or less (37,38). It is much easier to obtain a reasonably good value for  $K_m$  itself than to establish with much accuracy its change with temperature. Thus in most cases one can obtain the free energy of dissociation of the compound more easily than its heat of dissociation. The data which appear most self-consistent to this writer on this point are those obtained by Kiese (52), who determined the value of  $K_m$  for the carbonic anhydrase system at four temperatures. The plot of its logarithm against the reciprocal of the temperature yields an almost perfectly straight line whose slope corresponds to a  $\Delta H$  of 20,000 cal. This value, together with the value of  $K_m$  at 285.5°K.,  $5.2 \times 10^{-3}$ , gives an entropy of dissociation of 59.6 cal. per degree. Kiese calls the entropy of activation in this case -59.6 cal. per degree and thus assumes that the formation of the enzyme-substrate compound is equivalent to the formation of the activated complex. In view of his subsequent determination of  $\mu$  as 8900 cal. the picture does not seem quite so simple since these two values (i.e.,  $\mu = 8900$  cal. and  $\Delta S^\ddagger = -59.6$  cal. per degree) lead to a velocity constant of the order of  $10^{-7}$  in place of the experimental one of  $10^{-3}$  and the over-all entropy of activation is thus close to -40 cal. per degree. The activation process then is more than the mere formation of the enzyme-substrate compound.

Dann (15) has reported 13,400 cal. for the corresponding heat of dissociation in the case of the cucumber citric acid dehydrogenase system. This value involves measurements of  $K_m$  at only two temperatures but a large number of measurements were made at each temperature so that the averages should possess a fair degree of significance. Most of the lower values of heats of dissociation seem to be based on less justifiable considerations. Thus it is usual to state that, for the sucrose-invertase system, Nelson and Bloomfield (67) find  $\Delta H$  of dissociation to be 0 while Euler and Laurin find a value of about 2000 cal. We find that the data of Nelson and Bloomfield certainly do not warrant calculating any quantitative value for  $\Delta H$ , but, just as certainly they do not warrant assigning

a value of 0 to  $\Delta H$ . The data of Euler and Laurin do not permit a great deal of confidence in the value 2000 cal. for  $\Delta H$  except as a lower limit. Somewhat the same remark might be made concerning the work of Veibel and Eriksen (89). They measured  $K_m$  at 30 and 20°C. for the combination of emulsin with methyl- and ethyl- $\beta$ -D-glucosides. It is true that their data hardly justify use for evaluating a temperature coefficient, but, to exactly the same degree, the data do not justify the claim of no temperature coefficient (*i.e.*,  $Q_{10} = \text{unity}$ ). In fact if their data are treated analytically they rather indicate  $\Delta H = 7500$  cal. for the methyl glucoside and 4500 cal. for the ethyl glucoside.

The theory of Michaelis and Menten (62) applies more strictly to conditions of either fairly low enzyme concentration or fairly high values of  $K_m$ , or more generally to conditions in which  $E/K_m$  is small, where  $E$  is enzyme concentration. Straus and Goldstein (86) have examined the equilibrium behavior of enzymes and inhibitors or substrates more generally. They treat the kinetics of the reversible combination of one enzyme center with one molecule of substrate or inhibitor as a true bimolecular instead of a pseudo-monomolecular reaction. The ratio  $E/K_m$  is defined as a "specific concentration," and in noting the behavior of noncompetitive systems, they describe three zones of behavior depending on the magnitude of  $E/K_m$ , and thus on what simplifying assumptions are permissible in deriving a function relating the concentrations of the components of the system at equilibrium. In the intermediate zone of values of  $E/K_m$  the complete theory must be used, whereas for small values of  $E/K_m$  the enzyme is all combined, while for large values all the inhibitor or substrate is combined. Goldstein (29) has extended the treatment to competitive systems and finds that they show the same zone behavior as do noncompetitive systems. To test the predicted behavior the system cholinesterase-physostigmine-acetylcholine was studied. It was established that the inhibition of cholinesterase by physostigmine is competitive. A single molecule of physostigmine or acetylcholine combines with one center of cholinesterase. If  $K_i$  is the dissociation constant of enzyme inhibitor (physostigmine), and  $K_s$  the analogous constant for substrate (acetylcholine), for a temperature of 38° the following were the constants found, in terms of moles, liters, and seconds:

$K_i = 3.1 \times 10^{-8}$ , which analyzes into: the rate constants  $k_1$

(combination)  $= 1.4 \times 10^{+4}$  and  $k_2$  (dissociation)  $= 4.33 \times 10^{-4}$ .

$K_s = 1.25 \times 10^{-3}$ , analyzing to:  $k_1$  (combination)  $= 4.33$  and  $k_2$  (dissociation)  $= 0.0053$ .

The values of the rates for the cholinesterase-catalyzed destruction of inhibitor and substrate are, respectively:  $k_{DI} = 0.00003$  and  $k_{DS} = 58.0$ .

Reiner (69) has treated the case of multiple enzyme inhibition in which the inhibitor at sufficiently low concentration exhibited a stimulatory effect. Gottschalk has attempted to derive some principles underlying enzyme specificity in the field of carbohydrates by pointing out the substrate atoms or groups involved in the bonding with the enzyme. Evidence suggests that in  $\beta$ -D-glucosides and  $\alpha$ -D-mannosides, besides the oxygen atom of the glucosidic linkage, only the hydroxyl groups attached to  $C_3$  ( $\beta$ -glucosides) and to  $C_4$  ( $\alpha$ -mannosides) are available for contact with the enzyme protein. The specificity of enzymes acting on hexosides lies in certain atomic groupings arranged at the surface of the enzyme protein in a pattern able to attract the hexose residue by the glucosidic oxygen and one or more hydroxyl groups in a *cis* position to this oxygen. Thus a rearrangement of the hydrogen and hydroxyl groups at a carbon atom adjacent to an attachment group of the substrate invariably affects seriously the rate of enzyme reaction. When the same rearrangement takes place at a carbon atom more distant from the group in contact, the effect on the enzyme-substrate relationship is much less marked.

## VI. Entropy of Activation

Much less attention has been given to the interpretation of the entropy of activation than to the heat or the energy. Justification for and explanation of this neglect is possibly influenced by the following factors. From equations (3) and (9) we have:

$$\Delta S^\ddagger = R \ln (hZP/kTe) \quad (24a)$$

or:

$$P = (kTe/hZ) e^{\Delta S^\ddagger/R} \quad (24b)$$

and we usually are content to estimate a value of  $P$  without any general theory for it. In most cases the contribution of entropy

change to the free energy of activation is distinctly smaller than that of the heat change. Thus, although  $\Delta F^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger$ , we frequently find that  $T\Delta S^\ddagger$  is considerably smaller than  $\Delta H^\ddagger$ , so we tend to consider it negligible.

In a catalyzed reaction it may be stated as a rule of wide generality that any catalytic modification of a reacting system which increases the value of  $\Delta H^\ddagger$  for a particular reaction also increases the value of  $\Delta S^\ddagger$  for the same reaction. The two values rise or fall concomitantly. Now an increase in  $\Delta H^\ddagger$  will increase the free energy of activation while an increase in  $\Delta S^\ddagger$  has the opposite effect. Thus these two effects tend to mutually cancel each other and there will be a smaller change in  $\Delta F^\ddagger$  and thus in reaction speed than would be expected from the effect of the dominant factor alone. This dominant factor is so often the heat change that we tend to neglect the entropy change, yet in principle either may be the more important. In Table XI data for a few reactions are collected (83). These reactions are of little importance in themselves but they exemplify cases in which the effect of catalyst on the heat of activation is less than its corresponding effect on the entropy. Thus in the first example we find a catalyst which increases reaction velocity by nearly  $10^4$  times without cutting the heat of activation. In the second example there is the opposite effect. There the catalyst actually cuts down the heat of activation appreciably corresponding to about a fiftyfold increase in reaction velocity, yet the measured velocity *decreases* appreciably. The third case is similar to the second example. In example 6 the catalyst increases  $\Delta H^\ddagger$  by an amount which corresponds to decreasing the rate by  $10^{28}$  times, yet it is decreased only  $10^1$  times. In the other two examples the effect of catalyst is merely suggestive, since the temperatures of catalyzed and uncatalyzed reactions are not the same, but the data are just as striking. For example in case 5 the catalyst increases  $\Delta H^\ddagger$  by over 40,000 cal., yet the catalyzed reaction goes faster at 33°C. than does the uncatalyzed reaction at 60°.

In simple cases the theory for  $\Delta S^\ddagger$  may be rather clearly pictured, and their values may be approximated by estimation which is independent of rate measurements. To illustrate this we may use the data of Escolme and Lewis (18) on the acid hydrolysis of the peptide linkage in two related compounds, as shown in Table XII.



TABLE XI

Reaction	Catalyst	Catalyst concn.	pH	°C.	$k'$ , sec. <sup>-1</sup>	$\Delta H^\ddagger$	$\Delta S^\ddagger$
1. Xanthogenic acid decomp.	Alcohol	0.00 1.00	— —	25 25	$2.7 \times 10^{-7}$ $1.6 \times 10^{-8}$	13,290 13,670	-44.0 -25.4
2. Malic acid decomp by H <sub>2</sub> SO <sub>4</sub>	Water	0.00 0.93	— —	30 30	$4.8 \times 10^{-4}$ $1.2 \times 10^{-4}$	24,010 21,550	5.6 -5.3
3. Amylene addition to CCl <sub>3</sub> COOH	Acetone	0.00 1.04	— —	20 20	$1.5 \times 10^{-5}$ $5.5 \times 10^{-7}$	17,350 17,050	-21.3 -28.6
4. Egg albumen denaturation	Urea	0.00 0.6*	5.9 5.9	65 23	$2.5 \times 10^{-4}$ $3. \times 10^{-4}$	135,000 -3,000	325.0 -85.0
5. Oxyhemoglobin denaturation	Alcohol	0.00 30 <sup>b</sup>	6.5 6.5	60 33	$2.3 \times 10^{-4}$ $6.8 \times 10^{-4}$	76,500 120,100	154.0 319.0
6. Oxyhemoglobin denaturation	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.001 N 3.03 N	6.76 6.76	64 64	$3.4 \times 10^{-1}$ $2.4 \times 10^{-5}$	76,500 119,800	153.0 276.0

\* Gram per milliliter.

<sup>b</sup> Volume per cent.

TABLE XII  
ACID HYDROLYSIS OF PEPTIDE LINKAGE<sup>a</sup>

Substance	°C.	$k' \times 10^7$	$\Delta H^\ddagger$ <sup>b</sup>	$\Delta S^\ddagger(\text{meas.})$ <sup>c</sup>	$\Delta S$ (rot.)	$\Delta S$ (H <sub>2</sub> O)	$\Delta S^\ddagger$ (est.)
Acetylglutamine	60	3.36	21,184	-24.8	9.3	18.8	-28.1
Benzoylglutamine	80	0.85	21,024	-31.8	15.5	19.8	-35.3

<sup>a</sup> Values extrapolated (slightly) to unit hydrogen ion activity.

<sup>b</sup> Cal. per mole.

<sup>c</sup> Cal. per degree.

Here we have the same reaction in compounds so similar that the heats of activation are essentially identical and yet one goes significantly faster at 60° than does the other at 80°. Extrapolation to a comparable temperature indicates that there is a factor, some 25- to 30-fold in relative speeds, which is accounted for almost exclusively by the difference in  $\Delta S^\ddagger$ , which is seen from the table to be about 7 cal. per degree. The difference in the estimated values as shown in the last column is 7.2 cal. per degree. Stearn (80) estimated the value in the last column as follows:

$$\Delta S^\ddagger (\text{est.}) = -\Delta S (\text{rot.}) - \Delta S (\text{H}_2\text{O}) + \Delta S (\text{vib.}) + \Delta S (\text{solvent}) \quad (25)$$

The first term on the right hand side of eq. (25) takes care of the loss of internal rotations (or perhaps librations) when the activated complex is formed. The next involves the loss of entropy of the reacting water molecule. These two terms are the most important and the other two are neglected in estimating the values in the table. They come, respectively, from the vibrations which replace the lost rotations and from the decrease in the electrostriction of the solvent when the polarity of the system is "spread" through a larger volume in the activated complex. These two contributions should be nearly the same in both cases and it is seen that if they amount to about 3.5 cal. per degree the estimated entropy changes would be almost precisely those given directly by the rate data.

Other examples of a similar nature might be mentioned. In the cases of arbutin and phlorizin the reaction of the former involves a small positive activation entropy whereas that of the latter is -16 cal. per degree (64). This difference is largely due to the fact that, unlike arbutin, phlorizin has a heavy radical next to the bond

of the bridging oxygen, in the aglycone group. The internal rotation, or libration, of this group which is lost on activation, contributes over 11 units to the entropy decrease. An analogous situation occurs in the hydrolysis of the related substances  $\alpha$ -methyl-D-galactoside ( $\Delta S^\ddagger = 12.6$ ) and  $\alpha$ -methyl-D-galacturonide ( $\Delta S^\ddagger = -5.3$ ) (66). This difference is qualitatively explained in the same way if the ester bond cleaves at  $\text{RCO—OR}$  in place of  $\text{RCOO—R}$ , and these entropy values would themselves tend to confirm the conclusions of Kirmann (53) concerning the particular cleavage bond.

In the case of enzyme-catalyzed reactions there is an added consideration which has rendered the interpretation of calculated values of activation entropies very difficult and this is the variable and, for the most part, unknown standard state of the enzyme. There are very few data which have been obtained using enzyme preparations of known concentration. This means that the values of  $\Delta S^\ddagger$  calculated from older work are composed of two parts, a standard entropy of activation and an entropy of dilution or of concentration change from some unknown value to a rational standard state. Some ten years ago the present author reviewed the data then available (80) and obtained large negative values of  $\Delta S^\ddagger$  for all enzyme-catalyzed reactions. At the time he was aware in a qualitative way of this unknown entropy of dilution and stated (80, footnote p. 18) that: "when ordinary stoichiometry enters enzyme work with assured values, we shall find that the values of  $-\Delta S^\ddagger$  given here are too high"; however, at the time he had no idea how much too high they might be.

More recently two workers have obtained data on enzyme-catalyzed reactions in which the stoichiometric states of both enzyme and substrate are known and which thus permit standard values of  $\Delta S^\ddagger$  to be obtained. Butler (9) has studied the kinetics of trypsin and of chymotrypsin on various substrates. His findings, together with values for a few reactions catalyzed by hydrogen ion, are summarized in Table XIII. These entropy values are reasonably close to the values exhibited by reactions in which enzymes play no part.

The only other work known to the author consists of recent data by Laidler and Hoare (55), who studied the kinetics of the urea-urease system. They find that the rate of reaction is proportional to the first power of enzyme concentration. However, it varies with

urea concentration in a complex manner. From about 2 to 5% urea the rate is proportional to concentration, but above 5% the rate increases at first less rapidly, reaches a maximum, and falls. At high urea concentrations the *decrease* in rate is actually in proportion to the urea concentration.

TABLE XIII

Catalyst	Substrate	$\Delta H^\ddagger$ , cal.	$\Delta S^\ddagger$ , cal./degree
Trypsin	Benzoyl-L-arginine amide	14,900	-6.2
Trypsin	Chymotrypsinogen	16,300	+8.5
Trypsin	Sturin	11,800	-4.7
Chymotrypsin	Benzoyltyrosyl- glycyl amide	10,500	-17.4
Chymotrypsin	Pepsin	11,200	-11.5
Hydrogen ion <sup>a</sup>	Acetylglucine	21,200	-24.3
Hydrogen ion <sup>b</sup>	Salicin	31,300	5.6
Hydrogen ion <sup>b</sup>	$\alpha$ -Methylglucoside	37,600	19.4
Hydrogen ion <sup>b</sup>	Maltose	30,800	5.7

<sup>a</sup> Values of Escolome and Lewis (18).

<sup>b</sup> Values of Moelwyn-Hughes (64).

To account for this behavior the authors propose an extension of the Michaelis-Menten theory by suggesting the following explanation. The urease molecule has at least one pair of adsorption sites, and in the formation of the activated complex a urea molecule must be fixed on one site and a water molecule on the other site. At high urea concentrations the mass effect of the urea causes it to cover both sites and the reaction is inhibited. In the concentration range corresponding to rate proportionality with urea they find  $\Delta H^\ddagger = 8900$  cal. and  $\Delta S^\ddagger = -10$  cal. per degree, values not out of line with those in Table XIII. The hydrogen-ion-catalyzed decomposition of urea gave  $\Delta H^\ddagger = 24,500$  cal. and  $\Delta S^\ddagger = -14$  cal. per degree. In this system the energy of activation was found to be independent of *pH* between 5.8 and 7.8, but the rate of reaction was at a maximum at *pH* nearly 7, suggesting that the active catalyzing species is the zwitterion of the enzyme.

For proper insight into the mechanism of enzyme action as elucidated by kinetic data, much additional work of the nature of Butler and of Laidler and Hoare is necessary.

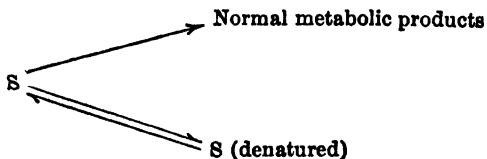
## VII. Pressure and Rate Processes

From equations (11) and (12) it is seen that the pressure effect on reaction velocity depends on whether or not there is a volume change when the activated complex is formed from the reactants. For simple chemical reactions as well as for other rate processes such a viscous flow of liquids, this volume change is not particularly large (82), ranging from a few milliliters to 12 to 15 ml. in many cases. For reactions involving larger and more complex molecules the volume change is often correspondingly larger and the pressure effect proportionately greater. Furthermore it is to be noted that, in the case of simple molecules, if they do not include ions in a highly polar solvent, a unimolecular reaction involves a volume increase when activation takes place, whereas a bi- or polymolecular reaction involves a decrease in volume (82). For large and complex molecules these generalizations no longer hold. Thus Campbell and Johnson (10) found a volume increase of the order of 50 ml. per mole when they studied the effect of pressure on rate of specific precipitation in the system rabbit serum and a trihaptenic dye antigen. This suggests the necessity of an unfolding process accompanying or preceding the chemical union of the components. Protein denaturation involves comparatively large volume increases, the value being specific for the reaction. Pressure is more effective in opposing the precipitation of purified human serum globulin than that of crystallized egg albumin (44). The globulin shows a volume increase of about 100 ml. per mole on activation (45). *Staphylococcus* antitoxin shows a progressively changing volume increase (50). For the first third of the denaturation process the value is about 40 ml. per mole, but has decreased to less than 20 ml. after 80% inactivation has been reached.

From some points of view, a study of pressure effects on reaction velocity may prove to be even more illuminating than that of temperature effects. Values of activation energy in some complicated process may vary in magnitude in a suggestive manner when reacting conditions are altered, but only rarely will they change in sign. The reason for this is that if a process is slow enough for its rate to be measurable the free energy of activation must be positive and only rarely will the entropy effect overshadow the heat effect. The contribution of  $P\Delta V^\ddagger$  to the free energy of activation may be to increase or decrease it without changing its sign, and thus it is

easy to study complex processes in which  $\Delta V^\ddagger$  not only changes in magnitude but may change in sign when environmental conditions are altered. As an example of such a complex process Johnson and Lewin have studied the effect of pressure on general metabolism of *Escherichia coli* (48,49). At low temperatures ( $22^\circ\text{C}.$ ) pressure increases the rate of disinfection as measured by the decrease in viable cells. This effect continues to pressures of 6000 pounds per square inch and would correspond to a net  $\Delta V^\ddagger$  of  $-76 \text{ cm.}^3$  per mole. At high temperatures ( $47^\circ$ ) where thermal disinfection is much greater, pressures up to 1000 pounds per square inch *decrease* or oppose the disinfection process. Beyond this value additional pressure has comparatively slight additional effect on the rate at a single temperature. There is, in this high temperature range ( $37$  to  $47^\circ$ ) a very marked pressure effect on the apparent activation energy of the process. The application of 5000 pounds per square inch lowers this quantity from over 130,000 cal. to about 30,000 cal. Johnson and Lewin take as a working hypothesis the view that the destruction of a single large molecule, such as a protein molecule, is symbolic of the single chemical event responsible for the logarithmic order of death of individual cells in the population. In the high temperature range this lethal reaction takes place with a large temperature coefficient and a large volume increase. The magnitude of  $\Delta V^\ddagger$  decreases from some 450 ml. between 1 and 67 atmospheres to a negligibly small value between 200 and 400 atmospheres at  $47^\circ$ . This suggests that at the higher pressures the activated complex no longer undergoes appreciable unfolding and thus many bonds which would be broken at low pressures remain intact. This would account for the large decrease in the activation energy.

A study of pressure effects on reaction rates, together with the recognition of the commonness of reversible denaturation as a property of proteins and enzymes (1), has permitted suggestions as to mechanisms of narcosis. Consider the scheme:



Johnson, Brown, and Marsland (42,43) point out that alcohol and certain other substances are known to influence the denaturation equilibrium, shifting it toward the denatured state. This effect should be abolished by increase of pressure. They find that the luminescence intensity of normal suspensions of certain bacteria, after first being reduced by the addition of any of a series of lipid-soluble narcotics, will be restored to its initial value by application of pressure. Such substances as ether, alcohol, chloroform, novocaine, ethyl and phenyl carbamates act in this way. Other substances such as barbiturates, sulfanilamide, etc. also decrease luminescence but in a manner not reversible by pressure and thus must act by a different mechanism. Johnson, Eyring, and Williams (47) have formulated mathematical expressions, extensions of equation (11), for the reversible inhibition of enzyme activity not only by temperature and pressure but also by substances which enter into reversible combination with the catalyst, the effects being mediated through an equilibrium normally present between active and denatured forms of the enzyme. They treat three possible types of enzyme-inhibitor combinations: (I) indiscriminate combination of inhibitor with native and denatured forms of enzyme; (II) combination of inhibitor with only the denatured form of enzyme; and (III) combination of inhibitor with only the active form of enzyme. Sulfanilamide conforms to type I behavior, as does *p*-aminobenzoic acid. The reaction of the former involves  $\Delta H$  of  $-15,000$  cal. and  $\Delta S$  of  $-35$  entropy units, a compound of one molecule inhibitor to one of enzyme, in its effects on luminescence of diverse species of luminous bacteria. The urethan inhibition of luminescence conforms to type II with  $\Delta H$  of  $-56,000$  to  $-70,000$  cal. and  $\Delta S$  of  $-165$  to  $-204$  entropy units, depending on the species. It seems that about three molecules of urethan combine with each enzyme molecule.

Eyring, Johnson, and Gensler (23) found that the heat inactivation of invertase is slowed up by increasing pressure by an amount indicating a volume increase of 69 ml. per mole. There was, however, a much smaller pressure effect on the inversion reaction itself.

The general process probably studied in greatest detail, so far as pressure effect is concerned, is that of bacterial luminescence, more particularly the system luciferin-luciferase in the presence of oxygen. Johnson and co-workers have opened a fruitful field (6,42,43,46,47). This work together with the general field of bac-

terial luminescence was completely reviewed in the 1947 volume of this publication (41). Eyring and Magee (24) applied the theory of absolute reaction rates to the available data. At low temperatures, pressure decreases the light intensity indicating a volume increase on activation. Above the optimum temperature, however, the net pressure effect is an increase in the intensity of luminescence. In this region the pressure is opposing the reversible inactivation of the enzyme which takes place with an even larger volume increase, so that the net effect is to increase the intensity of luminescence.

In all the data thus far reported, the reversible inactivation of enzymes or denaturation of proteins is associated with marked increases in energy, entropy, and volume. These changes represent for the most part an unfolding of the globular protein into the more linear fibrous forms.

On the basis of the study of combined temperature, pressure, and inhibitor effects on enzyme-catalyzed reactions Eyring, Johnson, and Gensler (23) have suggested the mechanism of such reactions as follows:

"If this unfolding is carried beyond a certain critical stage, or is accompanied by hydrogen bonding with narcotics such as alcohols, ethers, and similar polar bonds, the enzyme loses, at least temporarily, its power to take the shape appropriate for combination with the molecule to be catalyzed. The unfolding of one hundred or so amino acids is observed to result in a volume increase equivalent to about three-quarters of the volume of one amino acid.

"In general the folded enzyme must change its shape in the activation process to fit the molecule to be catalyzed, unless the active part of the enzyme is at the surface. Thus, bacterial luciferase expands about 50 cc. per mole in going from the normal to the activated state. This indicates a good deal of unfolding. Active invertase, on the other hand, shows practically no volume change in combining with sucrose to form the activated complex. This probably indicates that the active part of invertase is on the surface of the normal native molecule, so that very little change in shape of the enzyme is necessitated during the activation process."

### VIII. Long Range Action

While, strictly speaking, the recent work of Rothen (70,71) does not contain kinetic data it is of sufficient interest to be noted. He states that a pepsin or trypsin film will react on a deposited film of protein notwithstanding the presence of an intervening screen of inert material consisting of a formaldehyde-polyvinyl polymer.



This intervening screen may be very thick in terms of molecular dimensions if the substrate be properly deposited on the carrying surface. For example in a particular experiment it is reported that, while when four monolayers of bovine albumin are deposited by alternate successive immersion and emersion on slides covered with one double layer of egg albumen, an inert screen 90 to 100 A. thick prevents the inactivation of the albumin by trypsin, as evidenced by subsequent fixation of homologous antiserum; nevertheless if the same number of monolayers of bovine albumin is deposited on the slide by emersion only, a screen 100 A. thick permits complete inactivation of the bovine albumin, and a screen 250 A. thick still allows inactivation to a considerable degree. Apparently the enzyme is effective through a screen 50 to 60 A. thick regardless of the method of deposition of the substrate. If behavior of this nature is found to be general it means that in the usual systems, the formation of the Michaelis-Menten complex is not in itself the critical step in the specific activation process, though in particular cases it might represent the slow step in the over-all reaction. The idea would occur that the deposition of film is an orienting step which has its analogue in the formation of the enzyme substrate compound and that the specific catalytic action has another mechanism.

Some years ago Stearn (79) investigated the effect on the potential energy of a four atom reacting group (as for example the hydrolysis of a peptide bond) or bringing a dipole, properly oriented, close to the system. If this is brought too close there will be incipient mutual interaction among more than the four atoms, since those of the dipole will be involved, and serious interchange repulsions will result in raising the potential energy of the system. However, if the distance between the dipole and the reacting group is large enough to escape these repulsions and if the dipole is properly oriented, a distinct lowering of the potential energy of the reacting system followed, with a resultant catalytic effect. Some calculations were made on the basis of ordinary bond dipole moments and decreases in activation energy of from 4 to 16 kcal. were estimated as easily possible. However, these dipoles were acting through a dielectric of essentially unit constant and the separation from the reacting atoms was taken as only a few angstrom units. It is possible, however, that, as Rothen suggests, large long range forces

might act through greater distances. Such forces have been suggested by London (59). It will be of some interest if behavior of this nature is generally confirmed.

In connection with a study of gene structure Mazia (61) has been led to certain experiments in which enzymes have been incorporated with substrate molecules in protein fibers. These fibers he shows to be folded sheets so that the molecules incorporated presumably retain "native" properties. When pepsin is incorporated with egg albumen the fiber can be handled as long as the  $pH$  remains near the neutral point. Addition of acid to the same optimum  $pH$  shown by pepsin in solution results in a very rapid visible disintegration of the fiber and digestion of the protein.

While no kinetic figures are available, Mazia did study certain rates. He finds, for example, that the maximum rate of protein digestion comes when he incorporates about twenty molecules of substrate to one of enzyme in the film. The rate is not greatly different for a ratio forty to one but is much lower for a ratio eighty to one, although in all cases the final percentage digested is the same. Mazia also finds that 0.05 mg. enzyme incorporated in a fiber results in a more rapid fiber digestion than six times this amount of protein in solution. While these experiments are in no way analogous to those of Rothen, nevertheless there is also a possibility that in these fibers the enzyme may be effective in hydrolyzing protein molecules with which it is not in contact in the sense of a Michaelis-Menten complex.

The claim of specific long range forces acting through inert matter made by Rothen (70,71), has recently been questioned by Karush and Siegel (50a); they produce evidence, from electron microscope studies of deposited protein monolayers, that those on glass slides are not smooth layers of uniform thickness, but, possibly in drying, form ridges or peaks of 80 to 100 A., or in extreme cases 200 A., in height. These, they think, may project through the layers of inert screening material. Iball (40a) states that this assumption of Karush and Siegel is probably not justified since the screening material will probably stretch across the tops of any peaks or ridges. A more probable mechanism, he says, might be by crystallization of the screening layer from top to bottom, forming boundaries which might be penetrated by antibody groups.

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# PHOTOCHEMISTRY OF ENZYMES, PROTEINS, AND VIRUSES

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## I. Introduction

Since ancient times it has been recognized that light plays an important part in certain biological processes. Rollefson and Burton (292) believe that Stephen Hales (1677–1721) was responsible for the first investigation of photosynthesis which marked the beginning of photochemistry. After many isolated observations on photosensitive reactions, Grotthus (1817) and Draper (1841) recorded observations which led to the first law of photochemistry, namely, that only light absorbed can cause a reaction; its mere passage through a medium offers no catalytic effect. The absorption of light, however, may not result in a chemical reaction, and if it does the rate may not be directly proportional to the amount absorbed. The latter point was considerably clarified, in principle at least, by observations of Stark in 1908 and deductions by Stark and Einstein in 1912. Their conclusions are summarized as the second law of photochemistry, *i.e.*, in the primary process one quantum of active light is absorbed per molecule of absorbing and reacting substance. In a few simple cases, the ratio of the number of molecules reacting to the number of quanta absorbed, defined as the quantum yield or quantum efficiency, is unity. Secondary reactions may result in quantum yields higher than unity, *e.g.*, 2 to  $10^6$  for chain reactions, and sundry processes, to be exemplified below, are responsible for low yields.

In view of the action of ultraviolet (UV) light on living matter, resulting in sunburning and tanning (49), in death of bacteria, inactivation of viruses (170), production of lethals and mutation in higher plants and animals (89,138,324–326,338,339,356), it is worth while to study the photochemistry of the isolated cellular components involved. Strongly absorbing substances in tissues are pro-

teins and nucleoproteins. An appreciation of the photochemistry of proteins must be prefaced by a knowledge of the physicochemical changes which chemically related, low molecular compounds undergo following the absorption of UV radiation.

## II. Action of Light on Amino Acids and Peptides

### A. ABSORPTION SPECTRA OF AMINO ACIDS

A general statement of the subject of absorption by W. R. Brode, with some reference to these compounds, may be found in Volume IV of this series (p. 269). Additional material may be found in reviews by Anslow (12), and by Loofbrouow (216).

Most of the important amino acids may be regarded as derivatives of alanine. Alanine is expected to have selective absorption due to  $-\text{NH}_2$ ,  $-\text{CO}_2\text{H}$ ,  $-\text{NH}_3^+$ ,  $-\text{CO}_2^-$  and modified absorption due to interaction. Thus the photochemical behavior of an amino acid may be expected to have characteristics of amines, acids, and the corresponding zwitterions, depending on the conditions during irradiation. Amines show characteristic absorption as a band in the range 2100–2500 Å. (111,292). With fatty acids absorption begins at 2300–2400 Å. There is generally a maximum at about 2040 Å. followed by an increase at 1850 Å. and less. The apparent continuum throughout the range is attributed to the dimer (292). The absorption is supposedly that of  $-\text{CO}_2^-$ . A comparison of the absorptions of amino acids and their salts, with those of carboxylic acids, amines, and their salts has led to results which can most readily be interpreted in terms of dipolar ionic structure (92,208).

The ultraviolet absorption spectra of glycine (74,193,207,208,305), alanine (30,193,207,305),  $\alpha$ -aminoisobutyric acid (208), and valine (193,305) have been reported. Studies interpreted as indicative of zwitterion structures for amino acids in solution are given in references (204,209,350). General papers by Marenzi and Vilallonga (230) contain many of the important references and include spectra of  $\alpha$ -aminobutyric acid, leucine, isoleucine, serine, threonine, cystine, cysteine, methionine, glutamic acid, aspartic acid, lysine, arginine, proline, hydroxyproline, phenylalanine, tryptophan, tyrosine, diiodotyrosine, thyroxine, and histidine.  $\beta$ -Phenylalanine has been studied by numerous workers (2,17,193,305), as has tyrosine (116,158), and tryptophan (168). A comparative study of amino



acids with peptides and related compounds has been reported by Magill, Steiger, and Allen (228), and Allen, Steiger, Magill, and Franklin (11).

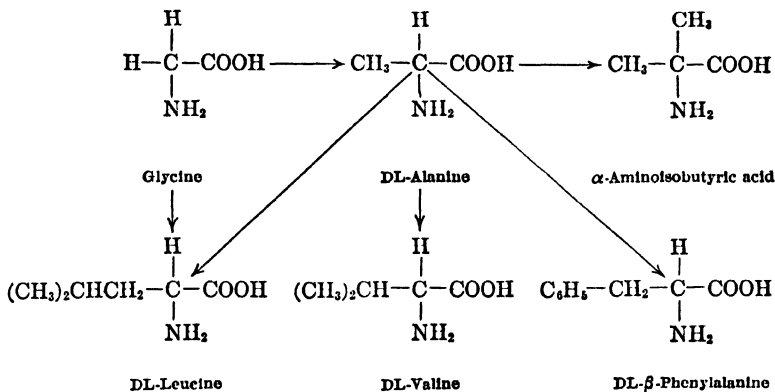
The simple aliphatic amino acids, *i.e.*, those not containing an aromatic nucleus, show end absorption only, rising from 2500–2200 Å. (216). An interpretation of the absorption spectra of glycine solutions has been offered by Anslow *et al.* (15). Primarily the study consisted of measurements of absorption from 6000 to 1850 Å. in water, dilute hydrochloric acid, and dilute sodium hydroxide. In water the possibilities are that there exists  $\text{NH}_3^+\text{CH}_2\text{COO}^-$  as monomer and polymer and  $\text{NH}_3^+\text{CH}_2\text{COOH} + \text{OH}^-$  and  $\text{NH}_3\text{OH}-\text{CH}_2\text{COO}^- + \text{H}^+$  ions. Hydrochloric acid solutions may contain  $\text{NH}_3^+\text{CH}_2\text{COOH} + \text{Cl}^-$  or  $\text{NH}_3\text{ClCH}_2\text{COO}^- + \text{H}^+$  and in sodium hydroxide solution  $\text{NH}_3^+\text{CH}_2\text{COONa}$  or  $\text{NH}_3\text{OHCH}_2\text{COO}^- + \text{Na}^+$  may be present. The absorption of glycine in water and in dilute hydrochloric acid are identical, however, so that the form in common,  $\text{NH}_3^+\text{CH}_2\text{COOH}$ , is most probably present to the greater extent in both solutions. In alkaline media continuous absorption indicates dissociation of  $\text{Na}^+$  from the zwitterion.

Alanine absorbs much like glycine, but with some shift toward longer wavelengths. The same is true for cysteine except that the shift is considerably greater (14). Aspartic acid, glutamic acid, butyric acid, and succinic acid are all similar to alanine in the region of continuous absorption. This is attributed to dissociation of the carboxyl groups to give  $\text{H}^+$  ions. The dicarboxylic acids show selective absorption in the region 4900–5260 Å.; this is attributed to enhanced vibration in the  $\alpha,\beta$  linkage (14).

The absorption of ultraviolet light by cystine is more like that of alanine than like tyrosine. This is of historical interest since it helped decide the controversy as to whether or not cystine was a cyclic compound (124). Cystine absorption rises sharply at about 3200 Å. whereas that of alanine does so around 2300 Å. in dilute hydrochloric acid. Continuous absorption indicates dissociation of the molecule. This probably leads to some polymerization as it does with glycine. In cystine the bonds broken are probably  $-\text{S}-\text{S}-$  as well as  $-\text{O}-\text{H}$ , according to Anslow's interpretation of the spectrum. Cystine shows a slight band of selective absorption at 2510 Å. also attributed to a weakening of the  $-\text{S}-\text{S}-$  bond. As a result of a speculative, theoretical treatment, it has been concluded

that in dilute hydrochloric acid cysteine chloride and cysteine are products of the reaction, whereas in water cysteine hydroxide and cysteine are formed. A study of the photochemical breakdown products of cystine has been performed (336b) and is summarized below.

In the important paper of Magill, Steiger, and Allen, the qualitative effects on spectra of modifying glycine have been summarized. Their scheme is reproduced below, with the arrows indicating an increase in the absorption of UV light. The effect may be attributed to mere weighting ( $-\text{CH}_2-$  added) and to the addition of chromophores exhibiting fine structure. The latter modification produces the more profound change.



SCHEME I

The absorption of light by L-tyrosine, DL-phenylalanine, and L-tryptophan is characterized by definite bands of selective absorption unlike that of alanine, leucine, histidine, proline, and hydroxyproline (Feraud, Dunn, and Kaplan, 116). Tyrosine shows a change of absorption with change in pH. In 0.1 N sodium hydroxide the molecular extinction coefficient is about 1.7 times as great as with 0.1 N acid and is displaced some 150 Å. toward longer wavelengths (168). The absorption of phenylalanine and tryptophan is not appreciably affected by pH changes. UV absorption spectra of tyrosine solutions have been determined at pH 1.35, 9.40, 10.0, and 11.21. Ratios of absorption constants at two wavelengths

TABLE I  
FINE STRUCTURE IN AMINO ACID SPECTRA\*

Substance	Absorption bands, angstroms											
	—	2415	2470	2540	—	2605	—	2672— 2680	2747— 2750	—	2816	—
Tyrosine	—	—	—	2540	—	2605	—	2672— 2680	2747— 2750	—	2816	—
Tryptophan	—	—	—	—	—	—	—	2694	—	2794	2804	2888— 2894
Phenylalanine	2350— 2366	2410— 2418	2462— 2466	2517— 2525	2574— 2576	2606	2635— 2643	2671— 2675	2715	—	—	—
Substance	$\lambda$ (min.), Å.				$\lambda$ (max.), Å.				Molecular extinction coefficient			
Tyrosine <sup>b</sup>	2450–2500 (2650)				2750–2800 (2850–2900)				1300–1600 200 (1200, 2200)			
Tryptophan	2425–2460				2750–2800				5100–6000 1000–2800			
Phenylalanine	2340				2560–2575				100–200 28			

\* Values from references (29,95,113,116,216).

<sup>b</sup> In acid. Values in parentheses are for alkaline solution.

plotted against  $pH$  give characteristic curves from which it may be calculated that tyrosine has three  $pK$  values, at  $pH$  2.6, 9.5, and 10.3 (121). The frequency at which continuous absorption of tyrosine begins agrees with the relation of Anslow and Foster (14) relating frequencies at which total dissociation is produced with the reciprocal of the square root of molecular weights of acids and which ascribes the carboxyl group as the seat of the energy absorption. The relation does not hold for phenylalanine and tryptophan.

Fine structure in the spectra of aromatic amino acids is summarized in Table I.

#### B. CHEMICAL CHANGES OF AMINO ACIDS INDUCED BY LIGHT

The most important effects produced by light in the UV region are (1) decomposition reactions, (2) rearrangements, and (3) sensitized reactions. Of these the first has received the most attention. With UV light there are associated with various wavelengths the following energies, in kilocalories per mole (the *einstein*):

Wavelength, Å.	Energy, kcal./mole
4000 .....	71
3130 (Hg) .....	91
2850 .....	100
2537 (Hg) .....	112
2000 .....	142

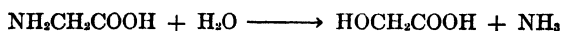
These values may be compared to bond energies to be found in proteins and protein derivatives:

Bond	Kcal.	Bond	Kcal.
C—C	80	C—S	55
C=C	150	C—H	87–100
C=O	145	C—O	70–82
C—N	45 (peptide)	C—Cl	75
C—N	66 (amine)	S—S	64

It is evident that if all the light is converted into vibrational energy at some bond in the molecule, sufficient energy is available at wavelengths shorter than 2850 Å. to break any single bond present.

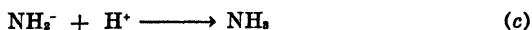
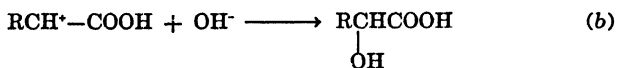
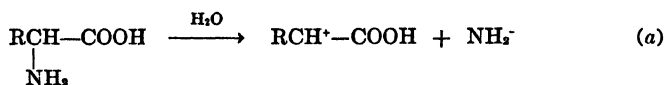
Some of the early findings concerning the action of light on amino acids may be found discussed in books by Heyroth (109) and Plotnikow (280), and a paper by Piffault (259). The classical work of Neuberg (246,247) involved the exposure of amino acids, peptones, and proteins to sunlight in the presence of oxygen and metal salts. The products observed were aldehydes, ammonia, and carbon dioxide. Similar studies were carried out with and without salts by Gopala and Dhar (141), by Roa and Dhar (283), by Ganassini (131) and by Benrath (40).

Irradiation of 0.1 *N* aqueous glycine solution by a mercury arc led to increases in conductivity and *pH*. This result was represented by the hydrolysis:



The important products were glycolic acid and ammonia. Side reactions were suspected because of the evolution of a gas rich in carbon monoxide (161). The reaction was most rapid at *pH* 3 and was at a minimum rate at *pH* 6. This change may be considered to involve an actual change in quantum yield with changing *pH* since the absorption of light by glycine solutions shows no such minimum (15). Wavelengths less than 2265 Å. are required to produce decomposition, although absorption begins at about 2400 Å. From the fact that methylamine absorbs in this region (160), it was concluded that the effective radiation is absorbed by the C—N linkage (357).

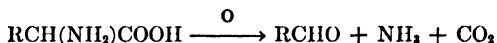
The photolysis of analine, betaine, and aspartic acid also shows minimum rates near *pH* 6. Since this minimum occurs near the *pH* at which zwitterion concentration is highest, it is concluded that the nonionic —CNH<sub>2</sub> group is involved in the photolysis. The photolysis, it was later suggested, proceeds by an ionic mechanism (359):



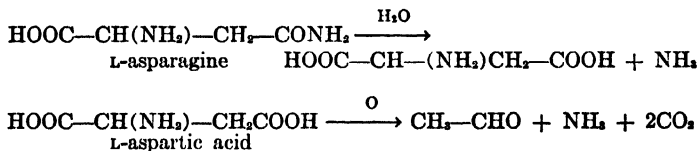
The rate would be expected to be higher at *pH* values other than near 7 since the products of step (a) are stabilized by (b) and (c). From optically active aspartic acid Weizmann *et al.* obtained race-

mic malic acid. This is indicative of an ionic (or free radical) intermediate. Lactic acid was isolated after irradiation of alanine. These workers also studied the breakdown of phenylalanine and tyrosine (turned yellow). Phenylalanine decomposes about 2.7 times as quickly as alanine in 0.1 *N* solution. This influence of the phenyl group may be due to two effects, increased light absorption and increased quantum yield involving a loosening of the C—N bond. From a study of the photochemical decomposition of phenylacetylalanines compared to acetylalanine, discussed below, it appears that the former reason is probably more important. Both phenylalanine and phenylglycine undergo side reactions which lead to the formation of insoluble substances. Quantum yields were determined for the photolysis of glycine (0.10) and alanine (0.087) based upon intensity measurements gleaned from a chloroacetic acid actinometer as a standard. These yields are probably more nearly 0.033 and 0.027 in view of a recent paper by Thomas (345), which showed that the quantum yield for chloroacetic acid is not unity as previously believed, but 0.33 to 0.34.

In the presence of oxygen, a photooxidation takes place (11,358) which may be represented by:



As mentioned above, L-aspartic acid can be hydrolyzed by UV light in the absence of oxygen. Both L-aspartic acid and L-asparagine are oxidatively decomposed upon being irradiated to yield acetaldehyde (37) (identified by absorption spectra). The former gives rise to a new absorption band at 2650 Å. (36). The net results are:



The second reaction as written is probably a summation since it would call for the simultaneous breakage of several bonds in the primary step. The primary photolytic process in irradiated organic molecules has generally been assumed to consist only in the fission

of one bond, *i.e.*, in the formation of two free valences per photolyzed molecule (43).

Two new absorption bands are produced at 2570 and 3130 Å. during irradiation of hydroxyproline solutions by a quartz mercury lamp (35). The irradiation of L-proline resulted in the appearance of a band at 3130 Å.

Splitting off of ammonia from a number of amino acids by light of the quartz mercury lamp has been measured semiquantitatively by Lieben and co-workers (213–215). The exposure was in open dishes so that photooxidation rather than photohydrolysis took place. 0.01 *N* solutions of various amino acids in 0.1 *N* hydrochloric acid in the presence of small amounts of photosensitizers (*e.g.*, rose bengale) were found to decompose to different percentages (Table II). The limiting wavelength for reaction in the presence of the

TABLE II  
RELATIVE YIELDS FOR DEAMINATION OF AMINO ACIDS (214)

Amino acid	NH <sub>3</sub> , % of α-amino N*	Amino acid	NH <sub>3</sub> , % of α-amino N*
Glycine .....	9.4	Lysine .....	20.9 (10.5) <sup>b</sup>
Alanine .....	10.4	Cystine .....	32.7
Aspartic Acid .....	7.3	Tyrosine .....	7.1
Arginine .....	9.4	Tryptophan .....	14.0 ( 7.0) <sup>b</sup>

\* In acid solution. Values in italics are in alkaline solution.

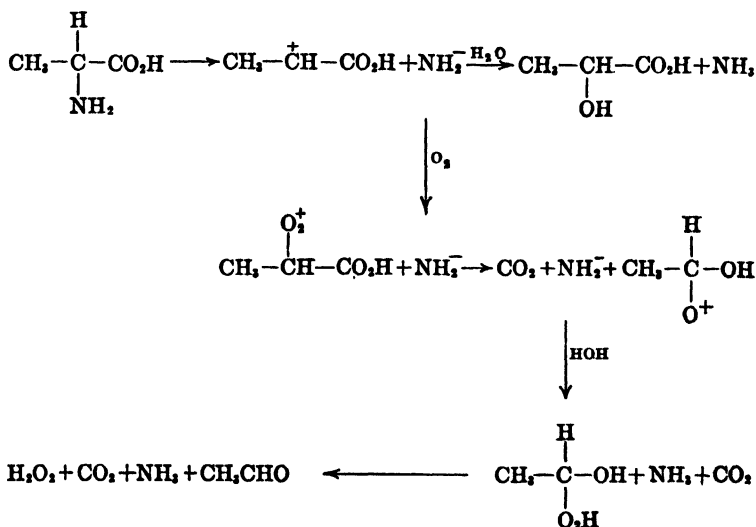
<sup>b</sup> Per cent total nitrogen.

photosensitizer is not known. Lieben and Urban ruled out decomposition of the guanidino group of arginine by a study of guanidine itself, which did not decompose. On the basis of the low quantum yield for methylamine decomposition (111) it is suspected that the terminal amino group of lysine might have not been involved. As may be seen, the photochemical rates for the deamination of α-amino groups from glycine, alanine, aspartic acid, arginine, and perhaps lysine (assuming that both amino groups are reactive) are about the same. Serine was also found to decompose during irradiation.

Irradiation of amino acids at various pH values by Lieben and Urban revealed that the rates were higher for glycine and alanine in acid media than in neutral and alkaline solutions in the presence of photosensitizers. It will be remembered that Weizmann *et al.*

found a minimum in neutral media but an almost equally rapid rate in acid or alkali, in the absence of a photosensitizer. Histidine did not show a significant dependence of decomposition on  $pH$ . The rate as measured by ammonia liberation was several times greater than for the others, probably through ring destruction.

Alanine has been exposed to UV light under oxygen and under nitrogen in a quartz vessel equipped with a cooling device (229). The source used was a Hanovia mercury vapor utility lamp without filters. This lamp has appreciable amounts of radiation at 2260 and 2399 Å. lines, *i.e.*, radiant energy corresponding to the long wavelength threshold found for the closely related compound glycine (2265 Å.). For a total time of irradiation of one hour of 0.01  $N$  solutions of alanine, in 0.01  $N$  hydrochloric acid, 28–30% of the total amino nitrogen was found as ammonia under a stream of oxygen and only 16–18% under a stream of oxygen-free nitrogen (six and five trials, respectively). After a correction for a blank (2%) it is clearly seen that the amount of photolysis under oxygen-free conditions is only half that under oxygen. A possible scheme is an adaptation of the one by Weizmann *et al.* (see above):



The primary process as written is the same in either system. Under



the influence of the light used, hydrogen peroxide, a possible product, is known to decompose. None is found under such conditions of irradiation (11).

Ellinger (107) found that irradiation of histidine hydrochloride solutions led to the formation of a pharmacological product with histaminelike action. Photocatalysts did not accelerate the rate of the conversion, which was unaffected, within certain limits, by the pH of the solution. Further irradiation at 2970 and 3020 Å. resulted in a loss in pharmacological activity. This work was confirmed by Holtz (174), who actually isolated crystalline histamine picrate. This decarboxylation is another path of photodecomposition of histidine in addition to ring rupture and deamination as observed by Lieben and Urban. Some evidence by Szendrő (336a) indicates that imidazole acetaldehyde is also formed from histidine.

The oxidation of cystine was reported not induced by irradiation (154). Szendrő *et al.* (336a), however, have reported that L-cysteine is formed when cystine is irradiated with unfiltered ultraviolet light from a quartz mercury arc in acid or alkaline solution. The product was identified by color reactions, optical rotation, a crystalline derivative, and conversion to cystine with iodine. The quantum yield is 0.02 (218a).

According to Harris (154), most of the oxygen taken up by irradiated plasma may be accounted for by the photooxidation of tyrosine and tryptophan. Berthelot and Amoreux (46) find that following exposure to the mercury arc indole-3-acetic acid is identifiable as a product from tryptophan. Photosensitizers such as hematoporphyrin increase the rate of oxygen consumption by aromatic amino acids in the UV and some action is even observable in diffuse daylight (210,211). Although such reactions may be important physiologically, they are difficult to interpret theoretically except in a general way.

Phenylalanine may take part in a series of changes similar to those of tyrosine (28). That phenylalanine solutions exposed to UV rays become pigmented has been noted by both Arnow (23) and Spiegel-Adolf (318). Irradiated neutral, acid, or alkaline solutions of phenylalanine were found by Arnow to give a positive Millon test indicating the formation of phenol. Furthermore, irradiated solutions give a color with nitrite-molybdate reagent indicative of dopa formation. Both Spiegel-Adolf and Arnow suspected

the presence of phenylacetaldehyde and Arnow was able to isolate a phenylhydrazone and to demonstrate a positive Schiff test. Even dry phenylalanine powder is partly destroyed during exposure to UV light.

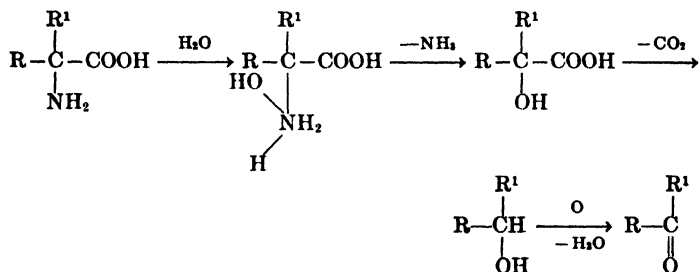
Tyrosine has also been investigated by Arnow (22). The destruction of tyrosine did not occur in the absence of air. In air, tyrosine is converted partly into 3,4-dihydroxyphenylalanine (dopa, identified spectroscopically) and partly into unknown products. Ammonia appears with irradiation but not aldehyde. It seemed possible to the worker that some of the melanin of the skin is derived from dopa by aid of dopa oxidase of the melanoblasts. The enzyme-induced polymerization might take place and be responsible for the latent period between erythema and tanning. An important difficulty with this hypothesis is that the oxidation requires the presence of molecular oxygen for the formation of pigment, yet pigment can be formed when the oxygen tension in the skin is considerably reduced during the time of irradiation. This pigment formation has been reviewed elsewhere (49). The oxidation of tyrosine by UV has also interested Rothman (293,294). Arnow has also followed the kinetics of the destruction of tyrosine in quartz tubes by light from a Victor therapy quartz-mercury, air-cooled arc. In the presence of an unlimited oxygen supply, the destruction proceeds as a first order reaction, i.e.:

$$T = T_0 e^{-k_1 t}$$

where  $T_0$  and  $T$  are the initial concentration and concentration of tyrosine at time  $t$ , respectively. The order did not vary with initial tyrosine concentration. No tyrosine was destroyed in a four hour period when tyrosine solutions were irradiated *in vacuo*. In a separate study dopa itself was also found to undergo a decrease in concentration with exposure; no tyrosine was formed. By combining the results of these two experiments Arnow calculated that one-third of the tyrosine molecules destroyed are converted to dopa and that the destruction of dopa is first order. The fact that tyrosine did not decompose *in vacuo* indicates that oxygen was present in the work of Weizmann *et al.*, who were not able to obtain a quantum yield for the decomposition of tyrosine owing to the formation



ulated above could be an intermediate. Stepwise the course offered by the worker is:



Although no attempt was made to identify isopropyl alcohol from  $\alpha$ -aminoisobutyric acid, ammonia was liberated and acetone was identified by a color test. The last step takes place in water without oxygen at wavelengths shorter than 2000 Å. (115). Incidentally, water alone did not give a positive test for peroxide after receiving light from the same source (Hanovia Alpine Sun Lamp) in a quartz test tube.

### C. ABSORPTION SPECTRA OF PEPTIDES

Magill, Steiger, and Allen found that the absorption of a dipeptide such as glycyl-DL-leucine is much greater than either or both constituent amino acids (228). Glycylleucine had been examined previously by others (1-4).

Replacement of an *N*-hydrogen atom of an amino acid or peptide by  $\text{CH}_3\text{CO}-$  has a bathochromic effect. The same may be said for replacement by  $\text{CH}_3-$ , but the effect is not large in either case. With the replacement of *N*-hydrogen by benzoyl ( $\text{C}_6\text{H}_5\text{CO}-$ ), however, the change is tremendous as is expected since most of the absorption may be assigned to the aromatic residue with its characteristic absorption bands. The absorption spectrum of an acetyl-amino acid is greater than the absorption by a mixture of equimolar amounts of the corresponding acids. The same is true for a mixture of an acetyl-amino acid and an amino acid as compared to the corresponding peptide.

The absorption of light by isomeric acetyl dipeptides was also studied with the finding that it was greater with the isomeride in which the amino acid of higher molecular weight occupies the ter-

minal position in the chain.

From the data of Magill *et al.* (228) and of Anslow and Foster it may be seen that simple amino acids absorb strongly to completely in the range of 2300–2000 Å. It may also be concluded that simple nonaromatic peptides absorb strongly to completely in the range 2400–2200 Å. (Fig. 1). It is thus not surprising to find that the —CONH— linkage may be cleaved at longer wavelengths than the region required for removal of —NH<sub>2</sub> from glycine (below 2265 Å.) (161). Introduction of the benzoyl group produces absorption from about 2800–2400 Å. with a rapid rise at 2600 Å. Benzene-sulfonyl- $\alpha$ -amino acids have also been studied by Magill *et al.* (228).

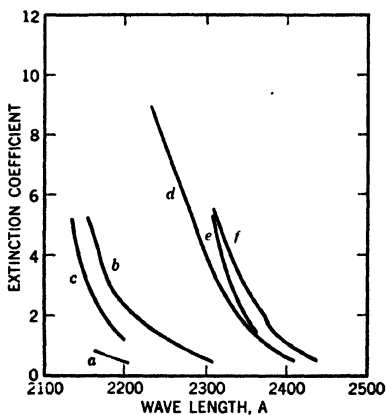


Fig. 1. Ultraviolet absorption spectra of (a) glycine, (b) DL-leucine, (c)  $\alpha$ -aminoisobutyric acid, (d) acetyl- $\alpha$ -aminoisobutyric acid, (e) glycyl-DL-leucine, and (f) acetylglycyl-DL-leucine (228). Solutions 0.05 *M* in water.  $\epsilon = (1/d) \log (I_0/I)$ .

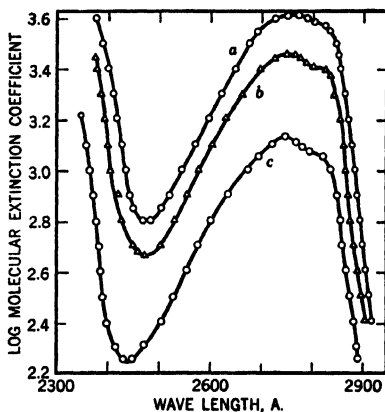


Fig. 2. Ultraviolet absorption spectra of (a) L-tyrosyl-L-tyrosyl-L-tyrosine, (b) L-tyrosyl-L-tyrosine, and (c) L-tyrosine (29). pH 4.5.

Barkdoll and Ross (29) have measured the absorption of light by L-tyrosine, tyrosyltyrosine, and tyrosyltyrosyltyrosine (Fig. 2). The found values of the molecular extinction coefficients were 1350, 2850, and 4160 at 2750, 2760, and 2765 Å., respectively. It is seen that the extinction coefficients are within 5% of the ratios 1/2/3. This correspondence plus the nearly identical position of the maximum is an example of the rule that chromophores separated by

one or more insulating groups, such as  $-\text{SH}_2-$ , absorb nearly independently (61). The slight shift toward the red with increasing number of residues is associated with an increase in size of the molecule. Similar results were observed by Fruton and Lavin for L-tyrosyl-L-tyrosine, glycyltyrosine, L-glutamyl-L-tyrosine, L-tyrosylglycine and glycyl-L-glutamyl-L-tyrosine (127).

Anslow and Lyman examined glutathione ( $\gamma$ -glutamylcysteinylglycine) (16). It has a 2800 A. band which is just resolvable into selective absorption at 2860 and 2760 A. These peaks shift to 3030 and 2920 A., respectively, in dilute alkaline solution. This was attributed to breakage of the peptide bond, the energy requirements for which are less in alkali than in water. At 2800 A. the energy requirements are 4.4 electron volts ( $\sim 102$  kcal. per mole). This energy is far above the normal bond energy of the C—N linkage. The absorption by glutathione was attributed to the amino acid residues which absorb as nearly independent chromophores. A band at 5500 A. corresponds to the spectrum of glutamic acid (14). Another band observed (2510 A.) indicates that some of the glutathione in solution was in an oxidized ( $-\text{S}-\text{S}-$ ) form. A broad band at 3250 A. is considered to be involved in breakage of  $-\text{SH}$  groups. At 2320 A. a continuum begins which was taken as indicating the presence of cysteylglycine. Some evidence for the existence of free glutamic acid was noted spectroscopically.

#### D. CHEMICAL CHANGES OF AMIDES AND PEPTIDES INDUCED BY LIGHT

For our purposes there are three groups of compounds containing the  $-\text{CONH}-$  bond, namely, simple amides, "hemipeptides" made up of aroyl, acyl, or arylacyl derivatives of amino acids, and true peptides composed of two or more amino acids. These categories will be treated separately.

In a preliminary note Mitchell (235) described the breakdown of stearic anilide as a monolayer on 5 *N* hydrochloric acid. The end products were stearic acid and aniline. No reaction was obtained at 2537 A. but at 2483 and 2375 A. measurable changes were produced. The principal reaction is probably:



Later Carpenter (70) also irradiated stearic anilide and identified

stearic acid as a product by film potential measurements. In this experiment the light source contained light of 2483 as well as 2537 Å. so that it was not possible, according to the worker, to decide definitely whether or not 2537 Å. light was effective. Unfortunately, in subsequent papers the photolysis was attributed to 2537 Å. (contrary to the results of J. Mitchell) without further published data (71-73). Under the same conditions Carpenter found that the weakest bond,  $\text{—C—N—}$  (48.6 kcal. per mole) (256), is also split in benzylstearylamine and  $\beta$ -phenylethylstearylamine. From this he concluded that a quantum may be absorbed by a phenyl group and transmitted through  $\text{—(CH}_2\text{)}_n\text{—}$  to the  $\text{—C—N—}$  bond with a resultant cleavage. A control compound, however, such as stearic methylamide, which also cleaves (290), was not studied. Only by comparison of compounds with and without aromatic groups, with a quantitative correlation between split molecules and quanta absorbed, can one hope to come to this or the opposite conclusion. The idea of energy transmission by  $\text{—(CH}_2\text{)}_n\text{—}$  is not consistent with the insulator properties exhibited by methylene groups in absorption spectra data.

TABLE III  
QUANTUM YIELDS FOR SPLITTING HEMIPEPTIDES  
 $\text{C}_6\text{H}_5\text{—(CH}_2\text{)}_n\text{—CONHCH(CH}_3\text{)CO}_2\text{H}$  AND  $\text{CH}_3\text{CONHCH(CH}_3\text{)CO}_2\text{H}$  AT  
2537 Å. IN AQUEOUS SOLUTION (206)

Compound	$\Phi$ , based on $\text{NH}_3$ liberated	$\Phi$ , based on alanine formed	Total quantum yield, $\Phi$
Benzoyl-DL-alanine	$2.09 \times 10^{-3}$	$1.25 \times 10^{-3}$	$3.34 \times 10^{-3}$
Phenylacetyl-DL-alanine	$2.27 \times 10^{-3}$	$4.77 \times 10^{-3}$	$7.04 \times 10^{-3}$
Phenylpropionyl-DL-alanine	$5.91 \times 10^{-3}$	$0.89 \times 10^{-3}$	$6.80 \times 10^{-3}$
Phenylbutyryl-DL-alanine	$16.9 \times 10^{-3}$	$26.8 \times 10^{-3}$	$43.9 \times 10^{-3}$
Acetyl-DL-alanine	$71.5 \times 10^{-3}$	None	$71.5 \times 10^{-3}$

In order to test the theory that the energy of the absorbed quanta can be propagated along an aliphatic chain to break a weak bond (70,290), acetylalanine was compared to phenylacetyl-, phenyl-, propionyl-, and phenylbutyrylalanine (206) at the suggestion of

F. M. Uber. In Table III are summarized results of the study of quantum yields for the photolysis of the series of compounds having the general formula  $C_6H_5-(CH_2)_n-CONHCH(CH_3)CO_2H$ . It will be seen that, contrary to the expectations of Carpenter, the quantum yield actually increases as  $n$  increases. The largest quantum efficiency is found with phenyl completely removed from the  $-CONH-$  linkage (acetylalanine). Thus, the phenyl group, although a strong absorber at 2537 Å., is, if anything, somewhat of a stabilizer for the splitting of the peptide bond in the sense that in its presence a greater fraction of the absorbed light is lost by nonchemical means.

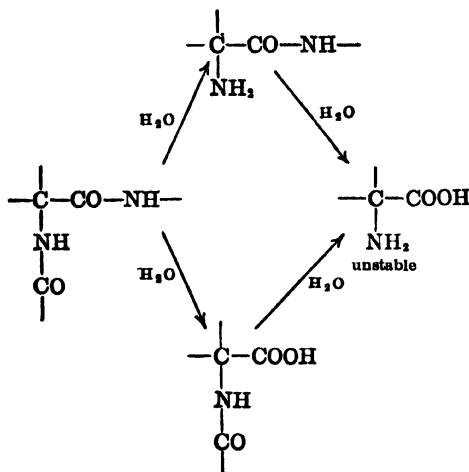
A more complete study of stearic anilide is that of Mitchell and Rideal (290). It was demonstrated that the relative quantum yield varies markedly with the molecular orientation at the interface of dilute hydrochloric acid-anilide and with respect to the direction of the incident light. The total aniline liberated was approximately equivalent to the stearic anilide decomposed photochemically. For the wavelength band 2350–2400 Å. the absolute quantum yield was found to be 0.26 ( $\pm 18\%$ ). The long wavelength threshold for photochemical hydrolysis was placed near 2500 Å. It was supposed that only single molecules are involved in the photochemical process because of a proportionality between reaction rate and light intensity (see below) and the magnitude of the quantum yield, which is of the order of unity. Absorption of light in the effective region studied probably occurs in the vicinity of the benzene ring and results in an electronic excitation. Since the yield is less than unity, some energy per mole may be lost via collisions or fluorescence, or may be rendered ineffective by a redistribution of the energy of excitation among the normal modes of vibration of the molecule. An observed dependence of the velocity of photohydrolysis on the mean ionic activity of the aqueous substrate suggests that the photolyte may be an ionization complex.

As is to be expected, benzoylglycine is very stable in sunlight (113,283). For early work on peptides papers by Hunecke (177), Abderhalden and Rosner (3,4), Asahina (25), and Guthmann *et al.* (150) may be consulted. That the decomposition products of glycyglycine were different from those of its isomer, asparagine, was noted spectroscopically by Becker (37). Alanylglycine is photolyzed at a rate between 2.6 and 4.2 times as rapidly as alanine in 0.05 to 0.1 *N* solutions (359). The free amino group was considered

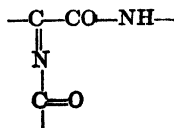


to be the principal source of the ammonia evolved. Work in this author's laboratory indicates that the quantum yield for liberation of ammonia from acetylalanine is of the same order of magnitude as the corrected value for alanine (0.087/3, because of erroneous value for chloroacetic acid actinometer, see above) and hence the increase rate could be attributed to the greater potential source of ammonia in the peptide as compared to alanine, assuming the rates for photolysis of the  $\text{—CONH—}$  link in acetyl- and alanylglycine to be the same.

25 derivatives of glycine and of secondary and tertiary amino acids have been exposed to UV light (Hanovia Alpine Sun Lamp) in quartz tubes by Allen *et al.* (11). Regardless of whether or not these molecules carried free  $\text{COOH}$  or  $\text{NH}_2$  groups, the substances underwent a degradation with the liberation of ammonia. To them this indicated a need for a hydrolysis prior to deamination. Their scheme for  $\text{—CO—NH—CR}^1(\text{R})\text{—}$  groupings is:



In the event that  $\text{R}$ ,  $\text{R}^1$ , or both are hydrogen, the dehydrogenated intermediate:



may occur prior to splitting of the —CONH— linkage. With both aqueous and alcoholic solutions of acetyl dipeptides the absorption curves are shifted toward longer wavelengths by irradiation with ultraviolet rays (Hanovia Alpine Sun Lamp) (Fig. 3). As a rule, the irradiation of amino acids, dipeptides, and related substances

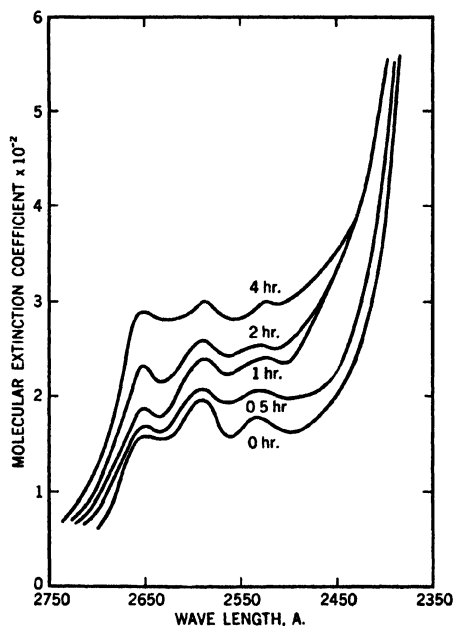
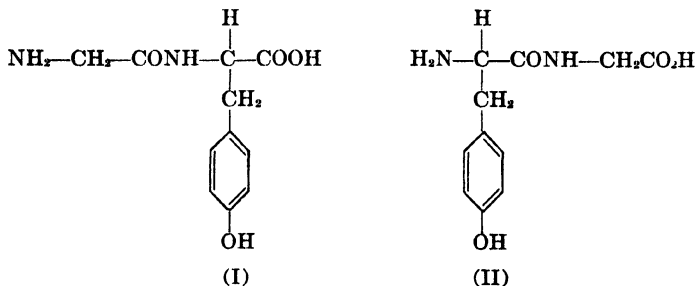


Fig. 3. Ultraviolet absorption spectra of acetyl-DL-phenylalanyl-DL-alanine, 0.01 *M* in ethyl alcohol, irradiated with ultraviolet light (11).

containing only aliphatic radicals causes increased absorption of wavelengths less than 2500 Å., while irradiation of those containing aromatic nuclei causes increased absorption of wavelengths greater than 2400 Å.

Without publication of experimental details, Carpenter has stated that glycyltyrosine and tyrosylglycine were both broken into glycine and tyrosine at 2537 Å. Alanyltyrosylglycine was split at both linkages. All three acids were identified by color reactions (73). It

would be worthwhile to find the relative quantum efficiencies for the splitting of glycyltyrosine (I) and tyrosylglycine (II), since



the stronger chromophore is on opposite sides of the  $\text{---CONH---}$  bond. Witte's peptone has been found to undergo profound alteration with respect to solubility and amino acid content on irradiation (212).

### III. Action of Light on Proteins

#### A. ABSORPTION SPECTRA OF PROTEINS

Of recent years many papers on details of absorption of light by proteins have appeared. Some of the more important papers are listed in the book by Ellis, Wells, and Heyroth (109). Ellinger gives a collection of protein absorption curves (108). Fine structure in spectra of a few proteins are tabulated in Table IV. Some typical spectra are shown in Figure 4.

That the absorption of UV light by proteins is largely a function of the unsaturated, ring-containing amino acids, tyrosine, tryptophan, and phenylalanine, was shown at an early date (155). Most of the remaining amino acids influence absorption at wavelengths of 2300 Å. or less. The absorption of blood serum has been shown to be caused principally by its tyrosine and tryptophan content (330). Later, it was found possible to account for the absorption curve of insulin on the assumption that in solution it behaves as a mixture of its constituent amino acids (195). With UV the changes in the absorption spectrum are exactly analogous to those of an irradiated mixture of tyrosine and cystine. In acid solution, in the range 2700–2800 Å., the absorption of insulin (which contains no tryptophan) agrees quite well with the calculated value based on

TABLE IV  
FINE STRUCTURE IN SPECTRA OF PROTEINS

Substance	Absorption bands, angstroms										Ref. No.
Gelatin	—	2529	2584	—	2644	2679	2745	—	2839	—	95
Horse serum albumin	—	2533	2583	2613	2645	2686	2733	2788	2847	2900	95
Egg albumen	—	2532	2587	—	2650	2680	2742	2799	2855	2923	95
Horse euglobulin	2487	2529	—	2616	2640	2680	2749	2795	2849	2915	95
Horse pseudo-globulin	—	—	2587	—	2649	2691	2747	2794	2849	2916	95
Insulin	—	2530	2586	—	2645	2683	2766	—	2839	2898	95
Pepsin	—	2520	2580	—	2650	2690	2760	—	2850	2900	202
Papain	—	—	—	—	—	—	—	2780 <sup>a</sup>	—	2900	127
Tobacco mosaic virus	—	—	2550	2600	2660	2690	2750	—	2820 <sup>b</sup>	2910	203

<sup>a</sup> Very broad band (2650–2850).

<sup>b</sup> Broad band.

tyrosine and cystine content (96). The absorption curve between the limits of *pH* 1 to 13 can be accounted for by its amino acid composition if a slight shift for tyrosine in the protein compared to that of the free acid is assumed. This behavior is partially in line with the empirical facts that in molecules two chromophores behave independently to a first approximation when separated by  $-(CH_2)_2-$  (20).

Soybean trypsin inhibitor and trypsin form a molecular compound the absorption of which is intermediate between the separate proteins (Fig. 4) (196) and proportional to the tyrosine and tryptophan content. The reaction between trypsin and the inhibitor may consist of the neutralization of free amino groups of trypsin by free carboxyl groups of the inhibitor. This bonding does not modify the absorption appreciably.

Absorptions by nucleoproteins, *e.g.*, tobacco mosaic virus and prolactin enzyme, exhibit a band at 2600 Å. which is attributed to purine and pyrimidine rings. Absorption of light by tobacco mosaic virus has been shown to be nearly equal to the sum of the separate

absorptions (densities) of the nucleic acid and protein components (303).

The influence of binding of the tyrosine phenolic group on absorption is notable in the behavior of bovine albumin (307) and negatively charged egg albumen (96). In egg albumen, unlike insulin, the phenolic groups apparently cannot ionize. With insulin, as the pH is raised from 9.9 to 13.0 a progressive change in absorption takes place with a shift of the maximum toward the red and an

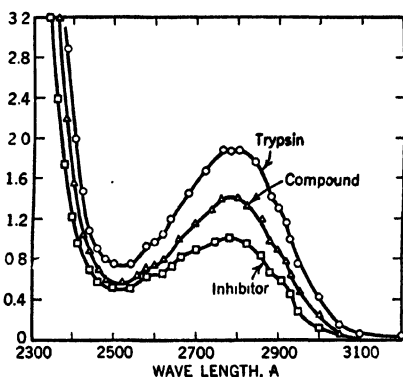


Fig. 4. Ultraviolet absorption spectra of trypsin, soybean trypsin inhibitor, and the trypsin-inhibitor compound (196). Ordinate is density per milligram protein.

increase in the extinction coefficient. The tyrosyl group is apparently able to ionize as a function of pH. If the pH of egg albumen solution is increased from 9.5 to 12 little change in the absorption spectrum occurs. At pH 13 the curve is quite different and the shift is not completely reversed by lowering of the pH to 12. Elevation of the pH to 13 results in denaturation and it may thus be concluded that in native egg albumen tyrosyl hydroxyl is bound, perhaps by hydrogen bonds to basic substituents, and cannot ionize to the extent that it does at the same pH in free tyrosine or insulin. It is also possible that in the native protein tyrosyl groups are not on the surface to react with hydroxyl ions. The former hypothesis is favored by the authors. Breakage of tyrosine bonds in egg albumen by UV light was also suggested by Crammer and Neu-

berger as a mechanism for denaturation. Chymotrypsin and soybean trypsin inhibitor behave in much the same way as does egg albumen; denaturation at high pH is accompanied by a sudden change in the absorption spectra of these substances (119).

The influence of pH on the absorption spectra of bovine serum albumin (Fig. 5) may be compared to that on the absorption of a mixture of amino acids in the same proportions as they are found in the intact protein (Fig. 6). The apparent binding of tyrosyl hydroxyl is evident below pH 10.5.

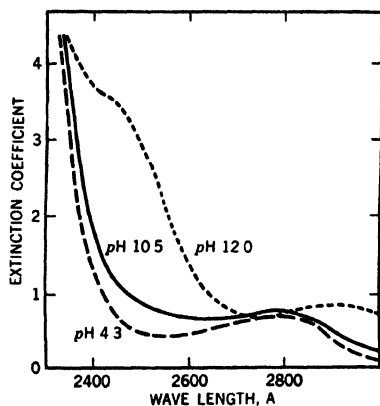


Fig. 5. Absorption in the ultra-violet of bovine serum albumin as a function of pH (307).

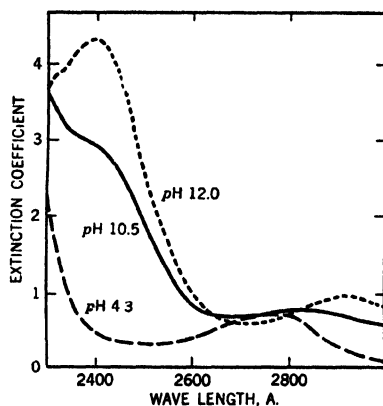


Fig. 6. Absorption as a function of pH of amino acids mixed in proportions existing in serum albumin (307).

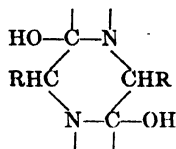
With papain Fruton and Lavin (127) found no spectroscopic evidence for tyrosine and phenylalanine; the entire band structure was attributed to tryptophan. When papain was hydrolyzed, however, the absorption spectrum was different from the original. During hydrolysis tryptophan was destroyed, which resulted in a loss of the 2900 Å. band. Two new bands appeared, namely, at 2740 and 2820 Å., which correspond to those exhibited by tyrosine. These experiments, the authors concluded, indicated that the absence of selective absorption of tyrosine in the absorption spectrum of a protein solution may not necessarily rule out the presence of tyrosine in the protein. Papain was found to behave as a mixture of tyrosine and tryptophan, according to Darby (97), who criticizes

the findings of Fruton and Lavin on the grounds of inaccurate observation and poor technique. Darby found that at higher pH values the specific absorption of tyrosine becomes more apparent and is readily resolved. The difficulties encountered by Fruton and Lavin may be involved to a lesser degree in the failure to obtain spectral results which check with chemical analyses in absorption measurements of the tyrosine and tryptophan content of proteins (167-169).

From the absorption properties of glutathione and of gelatin, which is poor in tryptophan and tyrosine, Anslow and Nassar (18) discovered that the peptide bond absorbs at 2800 Å. This specific absorption tends to be covered up by tyrosine and tryptophan in most proteins. Generally the bands of tyrosine and tryptophan overlap, giving rise to a wide band. With sufficient resolving power some proteins, such as egg albumen, give rise to curves which are not smooth but show definite structure. This structure may in part be identified with tyrosine, tryptophan and phenylalanine. In egg albumen two additional maxima, at 2840 and 2740 Å., have been assigned to the peptide linkage by analogy with the behavior of glutathione. Since the number of these linkages is great in a protein, a considerable proportion of the absorbed energy may be ascribed to rupture of this linkage. A similar statement applies to gelatin. The interpretation of Anslow and Nassar has been questioned on grounds of insufficient evidence by Crammer and Neuberger (96).

As a result of the frequent lack of quantitative agreement between summation of amino acid absorption and the corresponding protein, a general pigment absorption has been postulated by Goodwin and Morton for proteins (140). The subject has also been treated by Anslow (12,18). She and Nassar have concluded that general pigment absorption, which may have its origin in a long polypeptide chain fabric or the "cyclo-fabric" of D. Wrinch, may be found in gelatin and egg albumen. The fabric absorption observed is shifted in position by alkali as is the absorption of tyrosine (gelatin lacks tyrosine and tryptophan). It is proposed by them that a ring system bearing both hydroxyl and nitrogen is responsible. They also find that the end absorption (near 2400 Å.) of these materials is subject to shift by alkali in a manner typical of  $-C-C-OH$  residues. The same is true of amino acids but in proteins carboxylic groups are comparatively rare and the hydroxyl

residue is considered to be the more probable site of the alkali-sensitive absorption. Anslow favors the Wrinch cyclol structures from spectroscopic evidence:



This structure has been the brunt of much recent criticism, however (63).

It seems to the reviewer that part of the discrepancies between the summation of absorption by amino acid mixtures and proteins may be attributable to light scattering by the large protein molecules. Crammer and Neuberger do not consider this to be important for egg albumen (96). It is certainly true for viruses.

The theory of the absorption spectrum of protein monolayers has been partially developed by Mitchell and Rideal (237). It was shown that the molecular extinction coefficient in the monolayer,  $\epsilon_\lambda$ , at the wavelength  $\lambda$ , is related to the molecular orientation by the approximate relation:

$$\epsilon_\lambda = 3 \overline{\sin^2 \theta} \epsilon'_\lambda / 2 \quad (1)$$

where  $\theta$  is the angle between the axis of the effective molecular electric dipole and the direction of the incident light normal to the film,  $\overline{\sin^2 \theta}$  is the mean value of  $\sin^2 \theta$  for those values of  $\theta$  allowed by the usually small thermal agitation and orientational oscillation at any given molecular area, and  $\epsilon'_\lambda$  is the molecular extinction coefficient as measured in polar solvents. The number of quanta absorbed by the monolayer per square centimeter per second may then be computed for a quantum yield calculation as illustrated below.

#### B. DENATURATION OF PROTEINS BY LIGHT AND THE QUANTUM YIELD

The early work on the photochemistry of proteins, which has been reviewed by Spiegel-Adolf (316,319), Clark (88), Schomer (302), Brooks (62), and Arnow (21), has demonstrated that pro-



found physical and chemical alterations may be observed following irradiation by ultraviolet light. Examples are changes in optical rotation and dispersion coefficients, in ultraviolet absorption spectra, in viscosity, in  $pH$ , in surface tension, in electrical conductivity, in molecular weight, and in refraction of light. Some of the chemical changes directly observable as such are oxidation-reduction, ammonia formation, dehalogenation of halogen-containing proteins, destruction of benzenoid and heterocyclic rings, liberation of sulfhydryl groups, and loss of enzymic activity, if extant. In the past there has been little attempt to correlate physical and chemical effects with the amount of light absorbed. Only by the use of pure, preferably crystallized, proteins is this possible and only with enzymes and hormones is it convenient to do so. It seems quite probable that most of the early work does little to reveal the nature of the primary reaction only, in the denaturation process. Many of the gross changes listed above may be realizable only long after such delicate alterations as loss of enzyme activity occur. It seems most worth while to repeat many of the early studies with the aim of correlating loss of enzymic activity with structural and chemical changes. This is especially important in view of the "one hit" inactivation mechanism occurring in enzymic loss during radiation (221). The physical and chemical changes induced by light are considered in following sections. Wherever the change noted has been correlated with some specific alteration in the native state, *e.g.*, change in particle shape or loss of enzyme activity by a "one hit" mechanism, the point will be emphasized.

The denaturation of proteins by agents other than light has been reviewed recently (249). The authors point out that the changes proteins may undergo are so numerous and cover such a gamut of profundity that the term denaturation is ambiguous unless (a) the nature and magnitude of the changes are defined, (b) criteria for their recognitions are established, and (c) the agents which cause these changes are known. They state that: "Denaturation is any nonproteolytic modification of the unique structure of a native protein, giving rise to definite changes in chemical, physical, or biological properties." They exclude, for their purposes, processes leading to hydrolysis of peptide bonds. With light, however, this restriction must almost certainly be removed from a definition of denaturation since the evidence (235,237) indicates that in the

primary process peptide linkages adjacent to chromophore side chains become ruptured.

It seems, from the meager results reported, that the physico-chemical changes resulting in heat denaturation and radiation denaturation are different (88). The reversibility of heat denaturation under certain conditions has been established, *e.g.*, trypsin shows this phenomenon; however, Spiegel-Adolf states that light denaturation is not reversible (310,315). Heat denaturation occurs only in the presence of water but Stedman and Mendel (328), Clark (88), Fermi and Pernossi (117), and Agulhon (7) reported that proteins are denatured when irradiated dry. Heat- and light-denatured urease show different sedimentation schlieren patterns in the ultracentrifuge with a 50% loss in enzyme activity extant in both cases (227) (see below). In both cases both larger and smaller particles than the original were observed. A large change in the absorption spectrum of pepsin is observed following light denaturation; no such change is observed with heat denaturation (133).

A few experiments have been designed to show that the denaturation process induced by light is one of perhaps several steps. Bovie (51,52), Young (367), Galinsky (130), and Rajewsky (284, 285) consider a *preliminary* process in which the protein molecule becomes a new species (temperature independent) and a *physical process* resulting in a visible coagulum (highly temperature dependent). Clark divides the preliminary process into an initial production of photoelectric molecules (by emission of electrons) (82-85) resulting in an alteration of the protein, followed by a reaction between the radiation-altered molecule and water (87). In view of the systemic approach and the importance of Clark's findings, this work is summarized in some detail. At a *pH* removed from the isoelectric point, an irradiated protein may not precipitate upon being denatured and the degree of denaturation can only be determined by precipitating the denatured molecules with ammonium sulfate or by bringing the irradiated solution to the isoelectric point. In either event, it is assumed that the all-or-none hypothesis applies, *i.e.*, that all molecules in solution are native and all those which will precipitate with partial saturation or adjustment to the isoelectric point are denatured. This picture is in need of clarification. It seems possible that an enzyme might undergo loss of activity but fail to be precipitable until each molecule

has subsequently absorbed many more quanta, some of which may break additional bonds. The question can be put in the form: does the loss of activity parallel the loss of solubility? With pepsin Northrop was able to show that loss in activity with irradiation paralleled loss in solution *protein* nitrogen since the remaining active enzyme digested the inactivated pepsin. This is evidence that inactivation parallels denaturation (250). With chymotrypsin loss of activity initially parallels loss of solubility in half-saturated ammonium sulfate. Excessive irradiation results in further splitting of inactive protein with some resolubilization of inactive material (119). With a nonenzymic protein the question is not readily answerable.

Using a Tyndallmeter method Clark was able to show the following: Isoelectric egg albumen (*pH* 4.8) was irradiated at 4°C. with no precipitation. The solution was then brought to 40°, where flocculation began at once (at 40° the rate of heat denaturation and precipitation of native protein is negligible). Irradiation of the albumen was also performed at 14°. At 40° this irradiated protein precipitated out and the rate of flocculation at 40° was the same for both the 4 and 14° irradiated samples. Also, the final amount of flocculated material was the same in both trials if the total time of irradiation at the low temperatures was the same. Thus, the temperature coefficient of the light denaturation was unity. The process leading to flocculation after light denaturation has a high temperature coefficient, however. The rate is appreciable as low as 12°; the temperature coefficient was found to be 10.3. Similar results were observed by Rajewsky (284,286). Incidentally, albumen, heat denatured away from the isoelectric point, will precipitate at 4° if adjustment is then made to the isoelectric point. The temperature coefficient for heat denaturation flocculation was 14.3. It may be deduced that the molecule must be put into an active state by the absorption of a large amount of energy before coagulation takes place. This may even occur in the absence of water under UV. In the active state the protein can then react with water with a high temperature coefficient. The formation of the active state is looked upon as a physical step by Clark. The second, chemical, step may be similar to that of the first step in heat denaturation. The third step is the same for heat or light, namely a flocculation, presumably with a low temperature coefficient, as a

physical process. This last step perhaps involves diffusion as a rate-limiting step. This concept of three steps is consistent with the observation that a solution irradiated at pH 6.4 without flocculation, and then heated at 40° shows no opalescence, owing to pH. It has presumably passed through step 2, however, for upon lowering the temperature to 4° and the pH to 4.8 opalescence occurs. Negligible opalescence occurs after adjustment of an irradiated solution (pH 6.4, 4°) to pH 4.8 since the system has not passed through step 2. Therefore heating to 40° at pH 6.4 produces a permanent change in the light denatured molecule, which is necessary before step 3 if flocculation occurs. Mirsky and Pauling (234) suggest that the primary process causes a break in the native protein molecule which enables it to suffer denaturation at a temperature lower than usually required.

Clark also found the rate of light denaturation to be unimolecular. The significance of this point, indicative of a "one hit" process, is the following: With an exponential decline of native protein with time of irradiation at constant intensity, one may say that, although every quantum absorbed does not cause inactivation, when inactivation does occur the primary process involves only one quantum. The decrease in native protein ( $E$ ), with time for a "one hit" process is expressible as:

$$-d(E)/dt = k'(E) (h\nu) = k(E)I_0 \quad (2)$$

where  $(h\nu)$  represents the steady state concentration of quanta in the solution,  $h$  is Planck's constant, and  $\nu$  is the frequency of the light (120). The intensity,  $I_0$ , of the illumination may replace  $(h\nu)$  by suitably adjusting the proportionality constants  $k$  and  $k'$ . Upon integration there results:

$$kI_0 = (1/t) \ln (E_0/E) \quad (3)$$

where  $E_0$  is the initial concentration of native protein. Thus, in order to inactivate the same fraction of enzyme in two experiments with different intensities it is necessary that  $I_1t_1 = I_2t_2$ . This reciprocity rule would not follow if more than a single quantum were required simultaneously for denaturation. For all proteins for which quantum yields are available the rule has been found to hold. An example of the inapplicability of equation (3) is exemplified by a study of equine encephalomyelitis virus; the extinction coefficient

of the deactivated virus was considerably greater than that of native material (341). Relationships analogous to the above were deduced by Hussey and Thompson for the action of X-rays and radioactive radiations on trypsin and pepsin (178,179).

The reciprocity relationship was found not to hold exactly under conditions in which the time of exposure was related to the intensity by:

$$I/I' = (T'/T)^x$$

for a constant latent period before coagulation occurred,  $T_c$ . Here  $x$  was found to be equal to about 0.9 (137). This is not a direct method, however. Rajewsky found the expression:

$$T_c = K \left( 1 + \frac{1}{t} \right)$$

to hold, where  $t$  is the time of exposure, at constant temperature and intensity (284,285). Evidence with enzymes for which a measure of loss of activity was correlated with time and intensity illustrates equation (3) admirably (133,198,349,351). (See below.)

Clark has also studied the influence of urea on radiation denaturation of egg albumen with a General Electric Uviarc. Urea in sufficient concentration, proportional to the protein concentration, will prevent opalescence and precipitation of light-denatured albumen. It was suggested that the protein molecule may be enveloped in a shell of urea molecules which prevent flocculation.

The quantum yield (or efficiency) for denaturation,  $\Phi$ , may be defined as:

$$\Phi = \frac{\text{number of molecules denatured}}{\text{number of quanta absorbed}}$$

or:

$$\Phi = \frac{\text{number of moles decomposed}}{\text{number of einsteins absorbed}}$$

The second definition applies for monochromatic light for which the einstein equals  $N(h\nu)$ . The einstein depends upon the energy per quantum and is the energy associated with  $6.02 \times 10^{23}$  quanta.

In order to calculate the quantum yield for a protein one needs

to know the intensity of the source, the fraction of the light absorbed by native protein and the loss of native protein (or active enzyme) during the irradiation. For a protein without a specific activity such as virus, enzyme, or enzyme-inhibitor activity, for which a loss in identity cannot be followed quantitatively, the quantum yield would be quite nebulous in meaning. As it is, one assumes that the loss of, *e.g.*, enzyme activity or enzyme inhibitor capacity is concurrent with loss of the initial identity of the entire protein molecule in the native state. It is conceivable that some bonds may be broken in an enzyme without resulting in a loss in activity or that a number of bonds must be broken, only the last of which results in loss of activity. When activity is lost it is always found to be by a one hit mechanism. (It is probably significant, however, that Bull has concluded from acid hydrolysis experiments that the destruction of the protein molecule is initiated by the hydrolysis of a single peptide bond and the simultaneous activation of two or more peptide bonds is not necessary (65).) It is possible to calculate a quantum yield based on number of bonds broken, as for a protein monolayer, but analytical methods have not progressed sufficiently far to do this for proteins in solution. In the latter case first order decay would also be observed. Simple kinetic methods would not serve to distinguish between this stepwise process and one whereby the breakage of a single bond results in denaturation.

Two general types of behavior are encountered in quantitative studies with proteins, namely, cases in which the products of the primary inactivation step and reactants have the same molecular extinction coefficient and those for which the products have increased coefficients. For simple behavior the fraction of monochromatic light absorbed which is received by the native protein is given by (253):

$$\epsilon_R E / (\epsilon_R E + \epsilon_P P + \epsilon_S S) = d_R / d \quad (4)$$

for unit path length, where  $\epsilon_R$ ,  $\epsilon_P$ , and  $\epsilon_S$  are the absorption coefficients of the reactant, product, and solvent, of molar concentrations per milliliter of  $E$ ,  $P$ , and  $S$ , respectively. Neglecting the absorption of the solvent, which is usually low, being dilute aqueous buffer solutions, and finding that  $\epsilon_R \approx \epsilon_P$ , we note that the fraction of light absorbed which is received by active enzyme is, at any time,  $E/E_0$  where  $E_0$  is the initial concentration of reactant. Then:

$$\Phi = cf/MFI'_0 \quad (5)$$

where  $c$  = concentration in grams of reactant per cubic centimeter,  $f$  = fraction of molecules inactivated in time  $t$ ,  $M$  = molecular weight of reactant,  $I'_0$  = einsteins falling on the solution per unit time per cubic centimeter times  $t$ , and  $F$  = fraction of incident light absorbed by total protein corrected for the average amount absorbed by native reactant during the irradiation. The fraction,  $f$ , is determined by loss of enzyme activity, virus infective activity, enzyme inhibitor capacity, etc. Since, under the simple conditions assumed,  $E = E_0 e^{-kI_0 t}$ , the average amount of native reactant during the interval,  $t$ , is given by:

$$\bar{E} = \frac{E_0}{t} \int_0^t e^{-kI_0 t} dt \quad (6)$$

The function  $F$  is therefore equal to the fraction of the incident light absorbed by protein times  $\bar{E}/E_0$ .

An alternate form of equation (5), which is convenient to use in the special case of the approximate constancy of  $d$ , is:

$$\begin{aligned} \Phi &= \frac{E_0 - E}{I_0(1 - e^{-d}) t(\bar{E}/E_0)} = \frac{E_0 (1 - e^{-kI_0 t}) k I_0}{I_0 (1 - e^{-d})(1 - e^{-kI_0 t})} \\ &= \frac{E_0 \ln (E_0/E)}{I'_{ab} t} \end{aligned} \quad (7)$$

where  $I'_{ab}$  is the average number of einsteins absorbed by unit volume in unit time. This expression has been found to hold quite well if the inactivation is less than about 25% with proteolytic enzymes and holds exactly with tobacco mosaic virus. With the virus,  $\epsilon_B = \epsilon_P$  exactly (see below). If simple behavior is not observed, suitable corrections can of course be applied (226).

### C. LIGHT-INDUCED CHANGES OF PROTEINS AS STUDIED BY PHYSICAL METHODS

It is understood that the changes noted are associated with chemical alterations, although these alterations may range from the opening of hydrogen bonds (234) to the rupture of primary bonds.

### 1. Odor

It has been reported that proteins acquire a characteristic, burnt odor similar to that of skin exposed to an ultraviolet lamp. Gates noted that an odor of fresh urine or stale straw developed on irradiating crystallized pepsin in solution (133). Purified serum albumin, according to Mond (238,239) developed less odor than pure egg albumen. This characteristic is independent of the purity of crystallized egg albumen. A solution of egg albumen of pH 4.8 irradiated at 0°C. gave flocculation accompanied by a bad smell (18). The characteristic odor of irradiated serum albumin solutions mentioned by Pedersen (258) and by Stedman and Mendel (328) was not observed by Sanigar *et al.* (296). The latter workers attributed this to the precaution of rotating the irradiation tube, which ensured more uniform exposure and prevented the accumulation of degradation products of high light absorption in the layers nearest the source of illumination. The production of an odor is doubtless mostly a photooxidative process requiring considerable degradation beyond the primary denaturation step.

### 2. Color

All proteins, states Clark (88), upon being irradiated develop a yellow color which parallels odor production. Gates found a yellow coloration after a loss of 30–70% activity of pepsin by irradiation. From observations in this laboratory on solutions of pure amorphous insulin and crystalline urease, trypsin, chymotrypsin, pepsin, and tobacco mosaic virus, exposed to  $\lambda = 2537 \text{ \AA}$ ., it may be concluded that these changes do not parallel loss of specific activity, for no such color is observable at high percentages of inactivation. The virus does develop color when exposed to 2537  $\text{\AA}$ . at temperatures rising to 37° during inactivation. This change was also accompanied by turbidity. Neither the color nor turbidity arise during exposure at 25°. Inactivation takes place at both temperatures. Gates noted color production in his preparations of pepsin (133) and long time irradiated solutions of insulin are brownish (110). The nature of the compounds responsible for such color has yet to be elucidated. A yellow coloration may in part doubtless be ascribed to dopalike intermediates (22).



### 3. Refraction of Light

Serum albumin and pseudoglobulin (horse serum) exposed to UV light as 0.2% solutions in acid or alkali undergo negligible increase in light-refracting power (316). Results with an interferometer indicated degradation products in the supernatant liquids.

### 4. Optical Rotation and Dispersion Quotient

Proteins show increases in levorotation after radiation. This has been reported by Young (367) and Chalupecky (76) for egg albumen, by Stedman and Mendel for edestin (328) and gliadin, and by Ponthus for gelatin (281). The rate of change of optical rotatory power is probably always pH dependent.

When egg albumen is exposed to UV radiation at a temperature of 4° or less there results an increase in optical rotation and the increase is roughly proportional to the amount of light-denatured protein. The increase in optical rotation with light (or heat) can be due to structural or size changes in the molecules. Since osmotic pressure measurements failed to reveal a change in particle size there was no evidence of dissociation. One may conclude that egg albumen shows an increase in optical rotation as the result of structural changes (90).

Spiegel-Adolf (313) found the dispersion quotients (defined as the ratio between the specific optical rotation at Fraunhofer line F (4861 A.) and at Fraunhofer line C (6563 A.) for irradiated pseudoglobulin (2.2–2.9) to be different from that of heated solutions (1.88–2.00).

### 5. Ultraviolet Absorption Spectra

Spiegel-Adolf *et al.* found that the characteristic absorption bands of proteins undergo change with irradiation (157,310–312,320,322). The increase in absorption is more marked with albumins than with globulins.

With irradiation, the intensity of the 2800 A. band changes continuously, first becoming stronger and then weaker as a result of photolytic action (18,315).

It has been pointed out by Anslow (14,16) that with pepsin Gates found an appearance of strong absorption at 2500 and 3250 A.

following irradiation (133). These regions are characteristic of cystine and cysteine, respectively, and may be the result of liberation of these substances by prolonged irradiation (16).

A correlation of change in the absorption spectrum of pepsin with loss in activity induced by radiation has been offered by Gates (133) (Fig. 7). Irradiation did not greatly affect the peak at 2775 Å. or the final slope between 2475 and 2300 Å., but the absorption between 3100 and 2850 Å. broadened into the near ultraviolet and

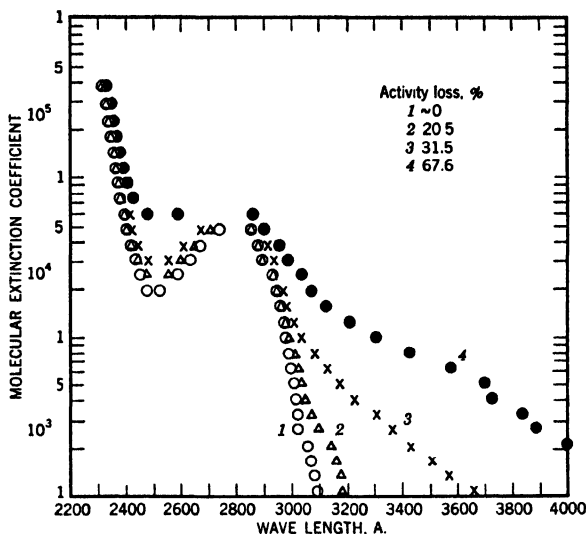


Fig. 7. Absorption spectra of pepsin solutions, pH 5.0, following loss of activity (133).

the valley at 2500 Å. filled up. Following the loss of 31.5% activity the pepsin solutions were yellow. Evidence for a difference in heat and light inactivation is afforded by comparing Figure 8 to Figure 7. Loss in activity by heat is presumably not accompanied by destruction of aromatic residues, as is postulated for light denaturation (see above).

If only products go over to reactants, with no further change in the reactants with continued radiation, it may be shown that a linear relationship exists between the amount of inactivation and the change in optical density:

$$c_0 - c = k(d_x - d_B)$$

Here  $(c_0 - c)$  is the concentration of products at time  $t$  and  $(d_x - d_B)$  is the difference between the optical density of the mixture at time  $t$  and the optical density  $d_B$ . It was found, by use of Figure 7, that this relationship did not hold for pepsin (133). It holds for chymotrypsin (119).

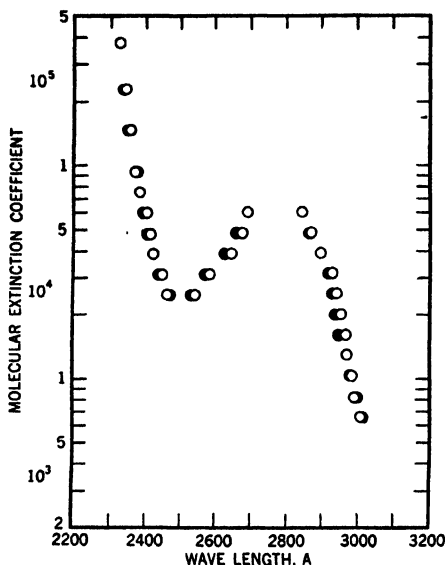


Fig. 8. Absorption spectrum of pepsin solution (open circles) compared to heat-denatured pepsin (43.5% activity) (133).

The extinction coefficients of egg albumen and ox serum were measured after irradiation in oxygen and nitrogen by Becker and Szendrő (38). Their results for egg albumen are shown in Figure 9. In nitrogen the extinction coefficient increases during irradiation with the maximum still present at 2800 Å. In oxygen the characteristic maximum disappeared, indicating photooxidation of aromatic groups. Chemical changes, listed under amino acids, above, of several aliphatic as well as aromatic amino acids can contribute to increased absorption on either side of the 2800 Å. absorption band.

An experiment similar to that of Becker and Szendrö has been carried out with chymotrypsin (119). The quantum yield is the same under oxygen or under nitrogen. At pH 3 and 8.6, at a residual activity of less than 1%, under nitrogen the absorption curves were of the same relative shapes and displaced upward between 2400 and 2900 Å., whereas under oxygen the curves showed considerably increased absorption at 2500 Å. and markedly reduced absorption at 2800 Å. In view of the constant quantum yield, it may be concluded that the photooxidative effects are not involved in the primary

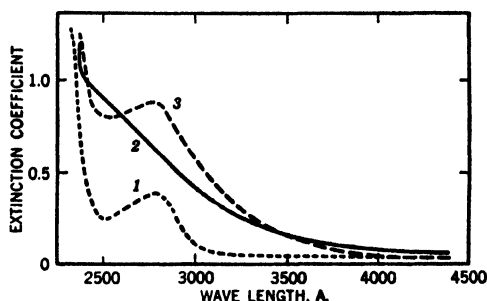


Fig. 9. Change in absorption curve of egg albumin after exposure to ultraviolet light (38): (1) control; (2) after irradiation in  $O_2$ ; (3) after irradiation in  $N_2$ .

process of inactivation. This is further borne out by the fact that at either pH virtually no difference in absorption is noted under oxygen or nitrogen with a 23% loss in activity.

Spiegel-Adolf also noted that proteins show increased absorption after irradiation in nitrogen and oxygen and concluded that light denaturation is not oxidative. This is not consistent with the results shown in Figure 9. These changes may not reflect the chemistry of the primary process of denaturation, however. For example, determination of the absorption spectra of irradiated solutions of crystallized trypsin, in air, revealed only a slight increase in extinction coefficient after the activity was reduced to more than 60% (349). With soybean trypsin inhibitor and chymotrypsin an increase in absorption of solutions exposed to air at both 2537 and 2800 Å. occurs progressively with denaturation. At 2537 Å., in acid

*pH*, the change is large enough to be used as a secondary method for the loss of active enzyme (119).

At both *pH* 5.4 and 7.4, human serum albumin shows a regular increase in light absorption as irradiation proceeds. The increase is general for 2400 Å. and greater and is most pronounced at the characteristic protein maximum. For the same radiation dose, the increase in light absorption is greater at *pH* 5.4 than at 7.4, which suggests that the reactions causing the increase are inhibited at the higher *pH* (296). Immediately after irradiation, solutions irradiated at 0°C. showed less increase in absorption at the protein maximum, but greater increase on the long wavelength side of the maximum, than solutions irradiated at 30° at the same *pH* (7.4) for the same length of time. Prolonged standing at 3° led to the disappearance of this difference, resulting in increased absorption below 3000 Å. and decreased absorption above 3000 Å. in both solutions but to a greater extent in the solution irradiated at 0°. This is consistent with the findings of Rajewsky (284,285), Clark (87), and Gates (133) that the temperature coefficient of the primary process is about unity with proteins. It also seems plausible that part of the absorption at wavelengths longer than 3000 Å. in solutions irradiated at 0° is due to an unstable intermediate stage (234), which produces, on standing in the cold (or warming), the structures responsible for the increased absorption at the protein maximum. Dialysis experiments on the two solutions gave results interpreted to mean that, while part of the increase in absorption results from dialyzable cleavage products, much of it must arise from reactions in the protein molecule without cleavage, or from cleavage products too large to diffuse through cellophane.

A solution of serum albumin of *pH* 7.4 after adjustment to *pH* 5.4 showed less absorption than one irradiated at *pH* 5.4. This indicated that the action of the light is inhibited at *pH* 7.4.

A large change in absorption spectra is that occurring during inactivation of ribonuclease (136). The trend may be seen in Figure 10 in which the optical density is plotted against time of irradiation at 2537 Å. After fifteen minutes, 52% activity remained. The irradiation was performed during exposure to air.

Harris (152-154) found that plasma takes up oxygen during irradiation, but since solubility and absorption changes can take

place in the absence of oxygen, *i.e.*, in nitrogen (311), it may be supposed that denaturation can take place without oxidation by gaseous oxygen. Furthermore, peroxides are not obtained from water at the wavelengths used on proteins and thus cannot be involved.

Too little is known about the absorption spectra of the individual amino acids and polypeptides and of their photolytic products to ascertain the nature of the reactions responsible for the increased

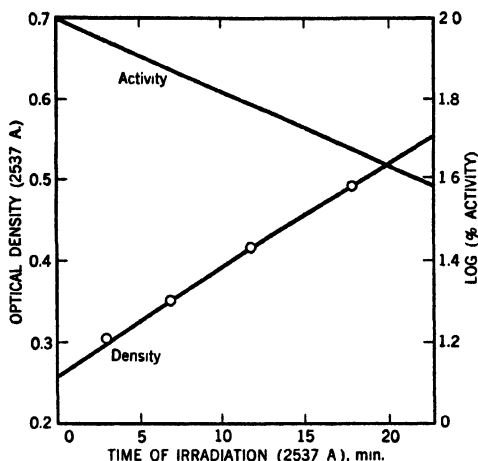


Fig. 10. Changes in optical density and activity of ribonuclease as a function of time of irradiation (136). Concentration of protein 0.87 mg. per milliliter.

absorption of light by irradiated proteins at the present time. Sani-gar *et al.* have shown that even if all the tyrosine and phenylalanine in serum albumin were converted to dihydroxyphenylalanine, the increase in absorption of albumin on irradiation could not be accounted for, and such a conversion is improbable (296).

The decrease in the rate of inactivation with irradiation of solutions of equine encephalomyelitis virus protein has been partly explained by the increase in absorption as denaturation proceeds (341). The more highly absorbing products give rise to an internal screening which decreases the statistical probability that the active virus will absorb quanta at constant incident intensities.

The UV absorption spectrum of tobacco mosaic virus (*i.e.*, at 2537, 2600, and 2800 Å.) does not change with complete inactivation at 2537 Å. (225).

### 6. Viscosity

The exposure of gelatin solutions to UV results in a decrease in viscosity (281). Similar results were obtained by Mond (238-240) with globulin and albumin solutions. These results could be explained by degradation of chains leading to less asymmetric particles or to a dehydration. The present status of the latter phenomenon as related to viscosity is still controversial (64). Increases in viscosity have been observed with euglobulins, pseudoglobulins, and albumins, in decreasing order, by Spiegel-Adolf (314). Young (367) recorded a rise in viscosity of irradiated ovalbumin. No such rise was found at pH 6.0 by Clark (85). An increase of viscosity has been interpreted as a change in the state of aggregation with irradiation (238,239). From viscosity theory, this could be true provided aggregation was accomplished by a change in particle shape. Tobacco mosaic virus completely inactivated by UV light (2537 Å.) showed no viscosity difference as compared to the original active sample (225). Electron micrographs of the inactivated material showed no change in particle size or shape.

Horse serum has been shown to undergo a linear increase in relative viscosity with time of exposure to a source emitting predominantly 2537 Å. radiation. The irradiated serum, unlike the control, revealed a reduction in viscosity with increasing external pressure which suggested an over-all increase in the asymmetry of the protein molecules in the irradiated serum (98). Serum globulin underwent a smaller change than did serum albumin for comparable exposures. Exposure times were in days; the change in viscosity, therefore, doubtless reflects secondary effects. It would be valuable to carry out such an experiment with an enzyme to find whether or not a change in viscosity parallels a loss of activity.

### 7. Surface Phenomena

For early work on this subject the review by Heyroth may be consulted (109). That UV light as well as light lowers the surface tension of proteins has been observed by Mond and Young. The same results have been obtained by Spiegel-Adolf (314) and Clark (85).

The decrease in surface tension, like the increase in viscosity is greatest in the euglobulins and least in albumins (314). Dognon and Gougerot (101,102) have studied surface films of serum albumin in the light of the mercury vapor lamp at pH 4-5. During the exposure the surface tension increases, at first quite rapidly, and ultimately approaches that of the substrate. At constant pressure the surface occupied by the film is decreased. Serum globulin, on the other hand, first shows a decline in surface tension followed by a rise which finally attains the value of the substrate; at constant pressure the radiation causes first an increase and then a decrease

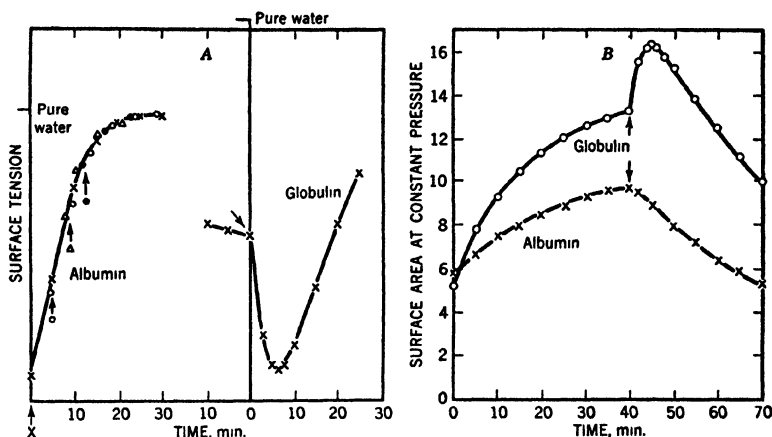


Fig. 11. (A) Variation of surface tension with irradiation. (B) Variation of surface area at constant pressure with irradiation time (102). Arrows indicate irradiation by ultraviolet light.

of the area occupied by the film (Fig. 11 A and B). Their interpretation is that UV breaks down albumins and globulins with the formation of soluble, less surface-active fragments. The albumins, however, spread more readily and the destruction is more immediately apparent. With globulins, which are incompletely spread, irradiation induces spreading initially, with a rise in surface area at constant pressure. These workers also found an increase in surface tension during irradiation of a gliadin sample of doubtful purity.

Cossu showed that with irradiation of serum albumin, the minimum of a surface tension - pH curve becomes a maximum at the



isoelectric point (94). Spreading of serum or egg albumin is partially destroyed by previous irradiation (248). A fully spread film can be caused to collapse partially by irradiation.

Mitchell and Rideal irradiated wheat gliadin, zein, and ovalbumin as surface layers (237). The layers used were carefully prepared monolayers, however. Their findings were that at 2537 Å. (2654 Å. and longer were not effective) egg albumen monolayers, which were originally solid, are liquefied and the phase boundary potential increases, as a function of the pH of the substrate, while following a pseudounimolecular law at constant area in conformance with theory. They suggested that the principal process was a photochemical hydrolysis of —CONH— linkages of the main polypeptide chain.

The modification of an interfacial potential difference produced by the presence of a monolayer was shown by Helmholtz to be given by:

$$\Delta V = 4\pi n\mu$$

where the change in potential,  $\Delta V$ , is caused by  $n$  molecules per square centimeter in the film-forming substance, each molecule possessing an average electric moment of effective vertical component,  $\mu$ . From the observed change of phase boundary potential on irradiation at constant area, the proportion of the total number of amino acid residues undergoing photochemical transformation can be deduced. The governing equation is:

$$\delta\Delta V_t/\delta t = KI_0(\Delta V_t - \Delta V_e)$$

$\Delta V_t$  and  $\Delta V_e$  are the phase boundary potentials characteristic of the film at equilibrium after irradiation for time  $t$  and at the end of the transformation and  $I_0$  is the intensity of the light falling on the unimolecular film (290). On account of the complexity of the chemical processes, the monolayer was illuminated for repeated short periods with intervals of darkness to allow the attainment of equilibrium. The total increase in phase boundary potential,  $\delta\Delta V$ , can be correlated with the amino acid analysis of proteins.

It is interesting to note, in connection with Mitchell's and Rideal's suggestion of hydrolysis of the polypeptide chains that no such changes were evident at 2537 Å. with monolayers of stearic anilide, their model substance. This is probably a result of the fact that with stearic anilide the aromatic residue is (a) directly on the —CONH— link and (b) it is on the nitrogen side of the link. In these respects stearic anilide as a model substance is of doubtful value especially since the wavelength 2537 Å. is capable of inducing virtually all the typical light-sensitive reactions of proteins. Its use did serve to show, however, that the orientation of the aromatic beam with respect to the direction of incidence can markedly alter the quantum yield.

The increase of phase boundary potential on illumination was

found to be consistent with the hydrolysis of about 8.2% of the total number of peptide bonds, which is close to the proportion of aromatic amino acid residues (7%). Mitchell and Rideal concluded that only those —CONH— groups adjacent to the aromatic side chains undergo photochemical hydrolysis at 2537 Å. Peptide linkages adjacent to tyrosine, phenylalanine, and tryptophan may undergo an oxidative photolysis the end result of which is hydrolysis. The free amino acids or their chromophoric residues pass into solution. This idea is supported by the quantitative total change in phase boundary potential observed for irradiated gliadin, zein, and ovalbumin (insulin films undergo partial solution), assuming no phase change during reaction and that solution of film is negligible, *i.e.*, only the chromophores liberated photochemically leave the sur-

TABLE V  
RESULTS OF IRRADIATION OF PROTEIN MONOLAYERS (236)

Protein	Wavelength	pH Substrate	Residues undergoing photo-oxidative transformation, %	
			Experimental	Theoretical (chromophoric)
Gliadin	2350–2400	2.0	6.0 ( $\pm 0.4$ )	5.7– 4.5
Ovalbumin	2537	2.0	7.0 ( $\pm 1.3$ )	8.3– 7.3
		7.0	5.7 ( $\pm 0.8$ )	
Zein	2350–2400	2.0	17.3 ( $\pm 2.0$ )	13.2–11.5

face. The total change in phase boundary potential is different, of course, for each protein. It was also speculated that energy absorbed in the chromophore groups can produce photochemical hydrolysis of those —CONH— groups separated by two carbon atoms and that aromatic groups can be expected to behave independently with regard to the law of photochemical equivalence (235).

A summary of results of the ultraviolet irradiation of protein monolayers (236) is given in Table V. It will be seen that a good correlation exists between the experimental values and the number of aromatic groups in the proteins.

Dognon and Gougerot (102) point out that their results with serum albumin and gliadin irradiated as surface layers do not agree with those of Mitchell and Rideal. The latter authors in general found a rise in surface pressure at constant area with irradiation,

that is to say, a decline in surface tension,\* whereas Dognon and Gougerot found an immediate rise of surface tension with their surface layers. This discrepancy does not seem resolvable without further study as to the nature of the layers, *i.e.*, degree of compression and thickness attained by these two groups of experimenters under their corresponding working conditions, and the influence of the "monochromatic light" used by Mitchell and Rideal versus the mercury arc of Dognon and Gougerot. Monochromatic light might expectedly give rise to split products of different (larger)

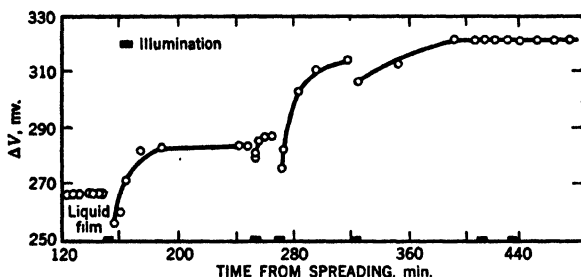


Fig. 12. Change in phase boundary potential of gliadin with intermittent irradiation (237).

sizes and solubilities than would the polychromatic light of the plain mercury arc; however, Mitchell and Rideal found that the total increase in phase boundary potential,  $\delta\Delta V$ , observed on irradiation is characteristic for each protein and appeared independent of the wavelength used.

Zein, insulin, and gliadin were also studied by Mitchell and Rideal. The change in phase boundary potential, in millivolts, with intermittent irradiation of gliadin on 0.01 *N*  $H_2SO_4$  at constant area with light of 2350–2400 Å. band width is reproduced in Figure 12. For the first few periods of irradiation, a fall in  $\Delta V$  is observed immediately after cessation of illumination and then a slow rise in the dark takes place to a value above that measured before irradiation. A constant value is finally obtained.

\* At constant area, the surface pressure,  $F$ , is related to the surface tension of substrate/air,  $\gamma$ , the tension of monolayer/air,  $\gamma_1$ , and the tension of monolayer/substrate,  $\gamma_2$ , by  $F = \gamma - (\gamma_1 + \gamma_2)$  (308).

By means of the molecular extinction coefficient in the monolayers,  $\epsilon_n$ , it was found possible to compute the quantum yield per chromophore for gliadin on the assumption that only aromatic chromophores are important in the primary photochemical changes. The number of quanta  $(I_0 - I)/h\nu$ , absorbed by the monolayer per second per square centimeter from a normally incident beam is given by (see above):

$$\frac{I_0 - I}{h\nu} = \frac{2.303}{6.06 \times 10^{20}} \epsilon' n \frac{I_0}{h\nu} \frac{3 \overline{\sin^2 \theta}}{2} \quad (8)$$

where  $n$  is the number of molecules absorbing per square centimeter.

By combination of this expression with the unimolecular rate law of reaction at constant area there is obtained the true quantum yield,  $\Phi$ , for the total photochemical change per chromophore. The yield is:

$$\Phi = kI / \left( \frac{2.303}{6.06 \times 10^{20}} \epsilon \frac{I_0}{h\nu} \frac{3 \overline{\sin^2 \theta}}{2} \right) \quad (9)$$

where  $k$  is related to the fraction of molecules decomposing per second and  $\bar{\epsilon}$  is the mean molecular extinction coefficient per chromophore (of phenylalanine, tyrosine, and tryptophan, the corrections for cystine, methionine, and arginine being negligible) as measured in solution. For gliadin,  $\Phi$  was found to be about 0.5 in the range 2500–2550 Å. This value is surprisingly high in view of the results for the photolysis of phenylpropionylalanine. Proposed explanations for such yields are presented under the topic of quantum yields in protein (see below). Quantum yields in solution are often lower than in the gaseous phase. Probably yields in monolayers will be found to be intermediate in value.

With insulin the results were modified by partial solution of the film on irradiation. The substrate beneath an irradiated (full mercury arc) insulin monolayer on 0.01*N* sulfuric acid gave a faint color with diazotized *p*-aminobenzoic acid which was indicative of tyrosine. The substrate gave no such color if a glass filter was used during the irradiation. The total amount of aromatic compounds liberated was, however, of the order of ten times that expected from the tyrosine content. The authors believed that histidine or photoxida-

tive products of tyrosine may have been responsible for these results.

Photochemical liquefaction of protein monolayers in the gel state is completely inhibited by small amounts of hydroquinone. It is concluded, therefore, that photochemical degradation in proteins involves an oxidative process as an essential step. Ordinary oxidation is not important since no detectable changes are produced in unilluminated monolayers by ozone or hydrogen peroxide, in the absence of ferrous ions, in the substrate. That photooxidation is a reasonable hypothesis is consistent with classical results (247), with the observed increase in amino nitrogen on irradiation of proteins (106,122,123) and with the ultrafiltrability of the pigment from irradiated ovalbumin (38).

In a discussion of the long wavelength threshold for proteins, Mitchell and Rideal state that no changes have been detected with the wavelength band 3125–3175 Å. or longer. This observation also provides evidence that the absorption spectrum of monolayers is not displaced toward longer wavelengths by more than 100 Å. from that in solution. Others have found a similar threshold wavelength limit with enzymes: for amylase, <2800 Å. (78); for lipase, <3300 Å. (78); for pepsin, rennin, invertase, amylase, catalase, laccase, and tyrosinase, <3022 Å. (7,8).

Unexpectedly fast reactions have been encountered in this wavelength region with gliadin and ovalbumin monolayers, supposedly because of photosensitizers. These high reaction velocities are reduced to a normal order of magnitude for these regions if potassium cyanide has been added. Presumably metallic ions are responsible.

Langmuir and Schaefer (199,298) have observed changes in viscosity of monolayers of pepsin after irradiation. Pepsin shows a "starlike" pattern when oil is placed at the center of a pepsin or water monolayer and allowed to spread. After irradiation at "2536 Å. and up," with 4% inactivation, the pattern was completely changed. With 25% loss in activity the pattern became round although the area which a given amount of protein covered remained the same. At the 32% remaining activity level the area covered was reduced to less than half. Correlation of inactivation by heat with change in pattern did not parallel the behavior with UV at 25–28% loss in activity; the heat-treated pepsin was still star shaped.

### 8. *Hydrogen Ion Concentration*

A number of investigators have observed changes in hydrogen ion concentration with irradiation. Acid solutions of albumins become less acid and solutions on the alkaline side of the isoelectric point become more acid according to Mond (238,239). Soybean trypsin inhibitor solution at pH 2.95 in 0.0025 *N* hydrochloric acid undergoes an increase to pH 3.45 after a loss of 30% inhibitor activity, at 2537 Å., at a concentration of about 2 mg. per milliliter (190). Clark found that irradiation of egg albumen with UV causes a decrease in pH (85). With the same protein Bernhart (44) observed an increase of over 0.3 unit at pH 3.94 and a decrease of 3.81 units at pH 9.03. Under N<sub>2</sub> a change of +1.28 units was found compared to -0.73 unit under air at an initial pH of 6.4. Such changes are to be expected; a detailed account is not possible now.

### 9. *Electrical Conductivity*

Gelatin solutions exposed to UV rays undergo an increase in conductivity (281). This is to be anticipated since the usual effect of UV is to cause depolymerization and liberation of ammonia.

### 10. *Molecular Weight*

Allusion to the work of Clark with egg albumen was made in Section 4, above. She found no evidence of dissociation in irradiated egg albumen by osmotic pressure measurements (90). The osmotic measurement may not be sensitive enough to detect dissociation, however.

There is reason to believe that particles in protein solutions are built according to a pattern which makes every atom in it indispensable for the completion of the structure. Svedberg and associates have accumulated data which indicate that the forces holding the large blocks of very big molecules together are less strong than the forces acting between the dissociable parts of smaller protein molecules. There is thus a possibility that one might observe the primary process of dissociation caused by the absorption of a quantum by using proteins of very high molecular weight (335). Hemocyanin of the snail *Helix pomatia* (molecular weight 6,740,000) in solution was exposed to radiation from a quartz mercury lamp. Using ultracentrifuge sedimentation determinations, Svedberg and Brohult found that at pH 6.2 a splitting into half-

molecules occurs. A further splitting of the half-molecules does not take place. Denaturation and finally complete precipitation followed prolonged irradiation. At pH 7.4 both halves and smaller sized particles were formed. It was concluded that light breaks the weakest bond in the molecule, the bond holding the halves together. It was found that the photochemical reaction occurs only at wavelengths shorter than 2800 Å., the region in which the protein part of the molecule absorbs. The region of absorption corresponding to the prosthetic group (3200–3700 Å.) is inactive. To further explore the assumption that this reaction is concerned with the primary process, frozen hemocyanin solutions were exposed to effective light at liquid air temperatures. The reaction rate was the same as at room temperature, with the formation of half-molecules. Such a low temperature dependence, i.e., a temperature coefficient of nearly unity, is typical of primary photochemical processes (336).

Unlike hemocyanin, hemoglobin undergoes a "pronounced in-homogenization," a continuous series of molecules of both lower and higher weight than the normal being found after irradiation at both room temperature and at 0°C. Irradiation at liquid air temperatures does not result in such degradation indicating that the effects observed when hemoglobin (and serum albumin) is irradiated at room temperature are attributable to secondary reactions. It will be remembered that Clark irradiated egg albumen near 0° and found that secondary changes occurred on warming the irradiated solutions, a situation differing from the present one in that secondary changes were not observed on warming the frozen, irradiated hemoglobin solutions. Apparently, the primary process requires the presence of liquid water in proteins, if only in trace amounts. ("Dry" proteins have been reported to undergo photochemical denaturation.)

K. O. Pedersen (258) has reported that, when serum albumin in solution at a pH of 3.5 was irradiated by ultraviolet light, the sedimentation constant doubled (indicating aggregation) and the diffusion constant showed a strong drift (indicating heterogeneity). Sanigar, Krejci, and Kraemer (296) also studied the effect of UV radiation on the behavior of serum albumin (human). They witnessed an asymmetrical broadening of the sedimentation curves,

which demonstrated the presence of particles heavier than native albumin and suggested the coalescence of molecules attacked by light to form aggregates. The liberation of substances of small molecular weight was indicated. From continuing radiation, progressive reduction in the peaks of the sedimentation curves occurs (Fig. 13) but in each series the sedimentation constants remain nearly the same as for the native protein. These data show that in the irradiated solutions the boundaries represent albumin of unchanged sedimentation constant and that the concentration of such albumin is diminished by successively longer periods of irradiation. In solutions sufficiently alkaline to prevent precipitation of the denatured protein, irradiation leads to the formation of increasing amounts of aggregated product of successively greater particle size; the size, however, seems to reach an upper limit. This type of aggregation seems to take place at low temperatures, or else very rapidly at 20°. In solutions near the isoelectric point, however, there is no limit to the particle size, and a visible precipitate is formed at the expense of aggregates of

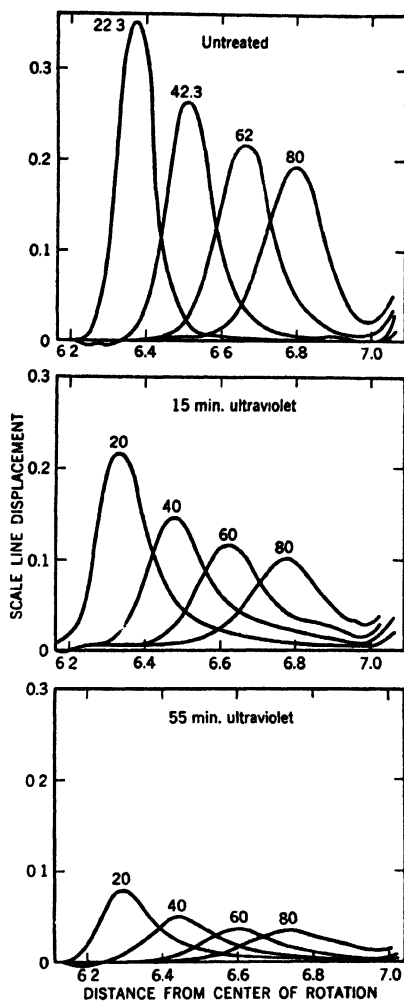


Fig. 13. Sedimentation diagrams of serum albumin, pH 5.44, after exposure to ultraviolet irradiation, at ca. 30°C. (296).



intermediate size. It appears that the aggregation process is inhibited at the higher  $pH$  (isoelectric point of serum albumin,  $pH$  4.9).

For equal irradiation ( $30^\circ$ ) the reduction in the concentration of albumin of unchanged sedimentation constant was found to be greater at  $pH$  5.4 than at 7.4. It might be concluded that the aggregation process is inhibited at the higher  $pH$ . It could also be concluded that less photochemical reaction occurred at the higher  $pH$  (lower quantum yield in the primary process, assuming little change in light absorption at the two  $pH$  values, which is true). The authors, however, found a correlation between reduction in albumin concentration and increase of light absorption with time of irradiation, which is fairly convincing evidence that both are manifestations of the same fundamental change (the primary process) and it was shown that the reaction causing increase of light absorption is inhibited at  $pH$  7.4. Thus the latter conclusion is almost certainly true; the former may also be true.

Results with the ultracentrifuge indicate that irradiation in part produces cleavage of peptide bonds. From irradiated solutions, from which no precipitation had occurred, it was calculated that not more than 4.5% of noncentrifugable material had been split off the protein. Complete hydrolysis of all aromatic residues in serum albumin would represent a loss of 6.6% as noncentrifugable material. With modification of about 70% of the serum albumin in this experiment (calculated from the sedimentation data) it is seen that the diminution of native protein is consistent with the view that only aromatic amino acids are hydrolyzed from the molecule.

Using horse serum proteins, albumin and globulin, Hollaender *et al.* (98) noted a decrease of colloid osmotic pressure with irradiation (mostly at 2537 Å.). This was interpreted to mean that light caused the formation of diffusible particles small enough to pass through the membrane. The smaller change was found with the globulin fraction. These workers also observed changes in electrophoretic patterns after irradiation of sera. Irradiation produced a progressive increase in homogeneity of the material with the formation of a new peak in the middle of the existing components. The new peak, D, was apparently formed from the other components in the proportions in which they are present. The D peak of a one-day-irradiated serum had the same mobility as that of a five-day-irradiated serum, even though a much higher proportion of the pro-

tein had been irradiated in the sample exposed longer. It was believed that the changes observed were largely due to a chemically homogenizing process, rather than to changes within the molecule. This process may involve (a) aggregation of the molecules in the proportions in which they are present, giving rise to final aggregates of the same charge density, or (b) simultaneous splitting and recombination of the molecules, giving rise to, not necessarily large, molecules having electrical homogeneity. From the fact that the serum had retained half its colloid osmotic pressure when it had become 90% homogeneous, and from other evidence for the splitting of proteins by light (38,44), it was concluded that (b) is more probable. Their interpretation is that denaturation by UV involves first

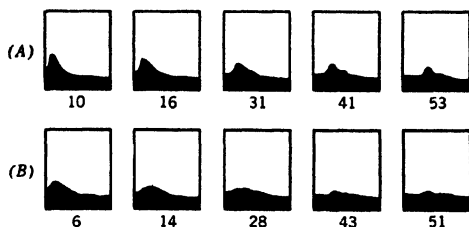


Fig. 14. Sedimentation behavior of urease (227). (A) Control run on 0.5% solution of urease, phosphate buffer solvent, pH 7.0. (B) Ultraviolet-irradiated sample, 55% residual activity. Numbers indicate minutes after reaching full speed, 30,240 r.p.m.

an unfolding, and then a splitting followed by an aggregation of the molecules to produce particles with a similar charge distribution but widely varying particle size. That a preponderance of large aggregates exists is indicated by the lowered colloid osmotic pressure (although some small particles may be lost by diffusion through the osmotic membrane).

Both heat and light caused an increase in viscosity and electrophoretic homogeneity but a much smaller decrease in osmotic pressure was observed following heat denaturation. Apparently molecular splitting is not so prevalent during heat denaturation. The changes observed in these studies have occurred by the absorption of an unknown number of quanta by each molecule on the average, and with the formation of an unknown number of broken

bonds. An independent procedure is needed to follow the loss of native molecules for comparison with the findings of the ultracentrifuge. This condition may be fulfilled if an enzyme is chosen for which the quantum yield for inactivation is known and which has a high molecular weight so that split products may be found in the sedimentation diagram. Urease has been used for this purpose (227). An aliquot of a 0.5% solution of crystalline urease was run in the ultracentrifuge at 60,000 times gravity (Fig. 14A). Another aliquot of the same solution was run after UV light (2537 Å.) had reduced the activity to 55% of the original sample. The light caused the enzyme to become extremely polydisperse and nonhomogeneous (Fig. 14B). The main component was present to the extent of 60% of the main component of the control sample; its average molecular weight was slightly decreased. Comparison also showed an increase in the amount of material both lighter and heavier than the main component. These observations suggest that irradiation brings about a primary process of inactivation and depolymerization followed by aggregation of the depolymerized material. Adjustment of irradiated solutions to the isoelectric point is known to augment this second step by inducing precipitation (296). In view of the fact that the quantum yield is low,  $\sim 10^{-3}$  (198), we may conclude that each molecule inactivated has been hit about a thousand times by light quanta. The possibilities are (a) that bonds at sites other than those involved in activity may be broken without loss of activity, (b) that a bond involved in activity is also a key bond which holds the molecule intact, or (c) that the integrity of the molecule as a whole is required for activity. In considering these possibilities (a) does not seem likely since this would not result in a 1/1 correspondence between residual activity and residual native material as determined by kinetics and the ultracentrifuge, respectively, i.e., no active, low molecular particles are observed; (b) is remotely possible if cystyl or glutamyl bonds are important to activity, since these may hold peptide chains together. *A priori*, it seems improbable, however, that such connecting links would occur only at sites of functionality, since the molecule would have to be "hollow" to have such a correspondence. On the other hand, point of view (c) has some credence in the classical all-or-none observations of denaturation, which has allowed us to calculate activation energies, heats of denaturation, and quantum yields.

This implies that molecules are either active (as enzymes) or inactive, with no partial activity attributed to some molecules or their denatured products.

The sedimentation pattern and constant of irradiated equine encephalomyelitis virus protein have been studied by Taylor *et al.* (341). The almost completely inactivated virus gave a sedimentation diagram essentially identical with that of the untreated control. Evidently major changes in the size of the protein molecules did not occur with inactivation by UV light (2537 Å.). No change in molecular size or shape was found (by means of the electron microscope) after inactivation of tobacco mosaic virus by light of 2537 Å. (230). Evidently the very high molecular weight, nucleic-acid-containing viruses are less susceptible to depolymerization than simple proteins.

### 11. Solubility

Serum albumin and pseudoglobulin (horse) precipitate quantitatively from electrolyte-free solutions by irradiation (316). Egg albumin, after exposure to UV light, shows a loss of solubility in water and will precipitate at the isoelectric point or on half-saturation with ammonium sulfate at a *pH* somewhat removed from the isoelectric point (52,85,87,328). All of its —SH groups are reactive. The long wavelength threshold is placed at 3000 Å. by Clark (88), who states that loss of affinity for water occurs without exception when proteins are exposed to UV light. If egg albumen is irradiated at 0°, the irradiated protein is not insoluble at its isoelectric point. The reactivity of its —SH groups has not been measured (19). Subsequently warming the irradiated solution from 0° to room temperature results in precipitation. Thus the denaturation has been separated into two steps, a photochemical change (at 0° or at room temperature) and a thermal change, which takes place in the protein modified by irradiation.

It is interesting to note that paramecia are not killed by short exposure to UV light at 18°. If such exposed paramecia are brought to 28° they die within an hour. Irradiation at 28° kills them also. If they are brought to 28° five hours after irradiation they do not die (54,57). In paramecia, but not in egg albumen, the effect of irradiation can, with time, either be reversed or abolished in some other way.

It has been postulated that the initial action of radiation lies in

the formation of photoelectric molecules by the emission of electrons (82,83). Clark believes that irradiation of a protein above its isoelectric point should cause a loss in negative charge of negatively charged molecules. Eventually such particles could become positive and result in precipitation with other, negatively charged particles. Below the isoelectric point the protein should be stabilized since all particles are positively charged. However solutions below the isoelectric point precipitated, after treatment with UV, more easily after addition of ammonium sulfate than did the other solutions, supposedly by combination with  $\text{SO}_4^{2-}$  ions. Experiments were compatible with the theory. Irradiation of chymotrypsin below the isoelectric point, pH 3, gives a precipitate, whereas at the isoelectric point none is formed in the absence of ammonium sulfate, however (120). It does not follow that coincidence of theory with fact is a proof in such a complicated problem. This photoelectric theory was advanced by Clark by analogy with the behavior of metals. It is not consistent with modern photorganic chemistry.

That breakage of the polypeptide chains with the formation of secondary protein derivatives occurs during UV irradiation is indicated by the results of Becker and Szendrő (38), Spiegel-Adolf (316), and Mitchell (235). The kinetics of formation of secondary protein derivatives under both air and nitrogen has been investigated by Bernhart with a precipitation method (44). Light-insolubilized crystalline egg albumin was precipitated by titration to the isoelectric point, pH 4.8, and weighed. Irradiations at pH 3.94, 6.4, and 9.03 all resulted in a lack of exact correlation between heat-coagulable protein and light-denatured protein precipitable at pH 4.8. The amount of protein insoluble at the isoelectric point reached a maximum after a few hours of irradiation and then decreased slowly. These results indicate that denaturation does not follow a simple first order process as described by Clark (87). By assuming denatured protein to be formed by a first order process and that protein derivatives, soluble at pH 4.8, are formed at a first order rate from denatured protein only, we may write:

$$dD/dt = k_1P - k_2D$$

Here  $D$  is denatured protein present at time  $t$ ,  $P$  equals native protein, and  $k_1$  and  $k_2$  are the rates of formation and photolysis of denatured protein, respectively. Since  $P = P_0 e^{-k_1 t}$ , where  $P_0$  means

total, undenatured heat-coagulable protein at time  $t = 0$  we may insert the second equation into the first and integrate. The result is:

$$P = \frac{k_1}{k_1 - k_2} P_0(e^{-k_1 t} - e^{-k_2 t})$$

The calculated values approximated the analytical values fairly well. After long periods of irradiation filtrates from the exposed, iso-electric protein solutions were found to contain protein degradation products which gave a positive biuret test and a precipitate with tannic acid or half-saturated ammonium sulfate. Little further precipitation was obtained upon saturation with ammonium sulfate. The filtrates contained both dializable and nondializable peptide-like materials. The velocity constants of denaturation (in solutions of pH 6.4) under nitrogen or air were nearly the same. The velocity constant for the formation of protein derivatives under air was over four times the value under nitrogen. The difference in the ratios of the constants of formation and denaturation in the two series, due presumably to presence or absence of oxygen, indicates that the reactions resulting in denaturation and formation of secondary protein derivatives are different in character. The denaturation reactions are seemingly independent of the presence of oxygen, whereas the secondary reactions are greatly influenced by oxygen. The ratio  $k_1/k_2$  was also found to be highly pH dependent in the presence of air, this is also indicative that denaturation and peptide splitting are different processes and do not proceed at identical rates.

### 12. Coagulation Temperature

The coagulation temperature of proteins is not a fixed property, being dependent upon a number of variables (80,81). Under similar conditions, however, the minimum temperature at which a precipitate first appears may be raised or lowered by irradiation. After denaturation, proteins, notably albumins, are in general more easily coagulated by heat (52,238,299,328). It has been reported that egg albumin and photosensitized fibrinogen after radiation no longer coagulate on heating under special conditions (58,85,175). The coagulation temperatures of globulins and fibrinogen are raised by exposure to UV (24,238).

### *13. Brownian Movement*

Gentner and Schwerin followed the coagulation process by observing the changes in the number of particles exhibiting Brownian movement (137).

### *14. Gold Sol Reaction*

Mond (238) found that irradiation of albumin solutions increases its protective power. No increase was found with globulin and fibrinogen. Spiegel-Adolf (316) offers evidence to show that proteases and peptones are produced by irradiating solutions of serum albumin and pseudoglobulin, which have ten to forty times the protective power of the originals. Gelatin lost its protective power and precipitated colloidal gold (317). She also found that the amino acids arginine, lysine, proline, and histidine flocculated gold sols after irradiation with UV light. In view of the formation of many new chemical species by irradiating amino acids, as discussed above, these findings are not surprising. The adsorption properties of the sol system are probably profoundly modified as aldehydes, hydroxy acids, and ammonium carbonate replace the amino acids. Since colloidal gold is a negative colloid, any increase of the basicity of a substance should increase its gold-sol-flocculating power. With proteins an increase in positive charge can result from destruction of carboxyl or by increase in amino groups. There are data in support of both.

## D. LIGHT-INDUCED CHANGES OF PROTEINS AS STUDIED BY CHEMICAL METHODS

### *1. Degradation*

The exposure of amino acids and proteins results in the liberation of ammonia (106,214). Solutions of globulin or blood serum irradiated with UV light contained nitrous acid, acetic acid, and imidazole derivatives, according to Holtz (173). Irradiated Witte peptone gave an insoluble product which no longer gave the biuret reaction. Tryptophan disappeared, the amount of histidine declined, and tyrosine was slightly destroyed (212). Lieben showed that tyrosine and tryptophan were destroyed when proteins in solution were irradiated (210,211).

The kinetics of the apparent destruction of tyrosine combined in egg albumin is not first or second order if measured by means of the Millon reaction. This has been explained by assuming (1) that tyrosine destruction is a first order process and (2) that tyrosine or a phenol is formed during irradiation (perhaps from phenylalanine) by a zero order process, and comparing the results of the assumptions to the data. It was found that oxygen promotes tyrosine destruction. Under nitrogen the rate of destruction of tyrosine was approximately half that obtained by irradiation in contact with air (45).

Carbon monoxide hemoglobin disappeared under UV light, according to Bénard *et al.*, who assumed that carbon monoxide was converted to carbon dioxide (39).

## 2. Sulfur Linkages

The —SH group of glutathione can be partially oxidized to —S—S— by exposure to UV (366). Kaemmerling (186) extended the work of Wels (360,361) on the reducing properties of irradiated ovalbumin solutions. Clear ovalbumin solutions saturated with nitrogen were found to liberate —SH groups, the reducing properties of which could be inhibited by reaction with moniodoacetic acid. The reagent was used to exclude the attributing of reducing power to the effects of radiation of glucose, glucosamine, phenylalanine, and histidine, all of which occur in ovalbumin and are known to yield reducing substances on irradiation (149).

Native proteins have either no reactive —SH and —S—S— groups or only a fraction of those potentially detectable. Denatured egg albumin has reactive —SH groups equal to the number of cysteine molecules in the hydrolyzate; the number of reactive —S—S— groups is equal to the number of cystine molecules in the hydrolyzate. This is true of proteins denatured by heat, trichloroacetic acid, urea, shaking in air, and UV light (232,233). The appearance of sulfhydryl groups in egg albumen has also been noted by Harris (156).

For studies on the detection of reducing groups after irradiation of proteins in sera the work of Schmidt, who used polarographic techniques, may be consulted (301). Wenig and Jirovec found an



increase in the —SH and —S—S— groups with irradiation (91). These groups are masked in the undenatured protein (363). Fiala showed polarographically (118) that UV light produces two parallel effects on proteins, namely a nonoxidative process produced only by short waves, and a photooxidation caused by either short or long waves in the presence of oxygen. It is believed that some oxygen becomes bound photochemically to the protein and some acts as a hydrogen acceptor with the formation of hydrogen peroxide.

The dyeing properties of wool are influenced by light (41,42).

### *3. Dehalogenation*

UV light will remove halogen from thyroxin, diiodotyrosine, Thyroidin, and artificially halogenated casein (211a).

### *4. Influence of Oxygen*

Experiments designed to test the influence of oxygen on the degradation of proteins by light are discussed under separate headings (Sects. III: C.11, D.2; IV: B.3,4,etc.).

### *5. Immunological Studies*

The effect of UV light on the immunological nature of crystalline urease has been studied by Pillemer, Ecker, Myers, and Muntwyler (260). Irradiated urease (and boiled urease) was unable to produce a specific antiurease in rabbits. Irradiated urease still had the ability to precipitate the serums from animals immunized with active urease, more so, even, than oxidized urease. It may be that the —SH group is the specific hapten, which is retained in irradiated urease but lost in oxidized urease. It seems possible to the reviewer that irradiation breaks mostly peptide bonds thus denaturing the enzyme, but without loss of —SH groups. The denatured enzyme is then subject to proteolysis in the animal body and is lost as an antigen.

Tubercle bacilli, vaccines, and some virus are rendered nonvirulent by light (2600 Å.), but they remain immunizers (201).

### *6. Photosensitization*

Proteins can be denatured by visible light plus a photosensitizer (83). It is not known whether this kind of denaturation can be divided into stages as with denaturation induced by UV light. Hemo-

TABLE VI  
PROTEINS IRRADIATED WITH ULTRAVIOLET LIGHT

Protein	Effects and remarks
Casein .....	O <sub>2</sub> absorbed (154).
Collagen .....	Becomes partly water soluble (344).
Edestin .....	O <sub>2</sub> absorbed (154).
Egg albumin .....	Absorption maximum at 2800 Å. disappears (39). Denaturation and molecular splitting causes formation of secondary protein derivatives which can be dialyzed at pH 4.8; O <sub>2</sub> does not influence the denaturation process but increases the formation of soluble protein derivatives (44). Tyrosine destroyed (45). Coagulation and odor (53). Denaturation consists of a stepwise process (see text) (85). O <sub>2</sub> absorbed (154). Aldehydes may be liberated (176,363). Number of sulfhydryl groups liberated equal to number in samples coagulated by typical denaturing agents (233). Redox potentials of solutions are reduced (244). Increase in UV absorption (322).
Gelatin .....	Loss of water swellability (60). Lowers surface tension (105). Ammonia liberated (106). O <sub>2</sub> not absorbed (154). Increase in electrical conductivity; lowers viscosity of solutions, decreases optical density (281,289). Viscosity of 1% solutions increases through aggregation (288).
Keratin .....	Intramolecular changes in spacing (26). Liberates sulfide ions (47).
Lens protein .....	Developed opacity (86).
Ox serum .....	No coagulation at 0°; becomes turbid in solution on raising temperature with clearing on lowering temperature (54,55).
Serum albumin .....	Undergoes change in surface tension behavior (94). Aldehydes are liberated (166). Coagulum differed from heat coagulum in not being alkali soluble (310). Increased UV absorption without shift of maximum and minimum with or without O <sub>2</sub> (320, 321). Coagulation depends on relative amounts of Na and Ca ions present (342).
Serum euglobulin .....	No increase of UV absorption in alkali (320).
Serum fibrinogen .....	Action of UV inhibited by hematoporphyrin (175). Stability of solutions increased (239,240).
Serum globulin .....	Stability of solutions increased (238-240).
Serum pseudoglobulin ..	Increased UV absorption in alkali (320,321).
Silk .....	Loss in tensile strength and elongation (231).
Trypsin inhibitor (soybean) .....	Inactivation by first order kinetics; quantum yield determined at 2537 Å. (190).
Wool .....	Liberates sulfide ions (47). Liberates SO <sub>2</sub> (31). Loss in strength and extensibility; increased swelling in acid or alkali (309). Increases solubility in alkali and somewhat in water; principal change is liberation of H <sub>2</sub> S (297,347).

globin is not denatured by visible light (48). It has been reported that serum albumin is denatured by visible light in the absence of a photosensitizer (367). This could well be re-examined, since it is incongruous with all other observations on wavelength thresholds mentioned in this review.

Table VI lists the proteins which have been irradiated. Significant findings are indicated in the second column.

#### E. DENATURATION BY LIGHT WITH ACETONE, ALCOHOL, AND ELECTROLYTES PRESENT

Denaturation by light, heat, alcohol, and acetone apparently have in common a dehydration of the protein (21). The mechanisms whereby the dehydrations occur are doubtless different. Mond found that the amount of alcohol required to induce precipitation of albumins was less after irradiation (238). The addition of acetone or alcohol prior to irradiation hastens the "denaturation process" (300,367). Coagulation of egg albumen is much more rapid in the presence of ammonium sulfate (51,367), sodium chloride, or potassium thiocyanate (367).

#### F. SUMMARY

In the past the bulk of work in this field has been on a low, empirical level of research. Experiments have not been designed to test theories but rather just to see what happened to a given measurement, *e.g.*, conductivity, sedimentation velocity, surface tension, during or following an exposure. Consequently much of the data is curious rather than instructive. Up to the late thirties little information was available which was amenable to clear-cut conclusions concerning the primary action of quanta. At present we can draw only the crudest over-all picture of what takes place during irradiation. A few generalizations are probably valid, however, and may be formulated at this time. Denaturation-inactivation occurs as a one hit process, although many quanta ( $10^2$  to  $10^4$ ) may be absorbed on the average by each molecule prior to inactivation. Probably the absorption of light of less than 3000 Å. by any chromophore can give rise to denaturation. The quantum yield for cleavage of the peptide bond is apparently greater on monolayers than in solution although no comparison with model substances was made at the same wavelengths. Calculations indicate a value

of unity for denatured proteins as monolayers whereas in solution low values for inactivation-denaturation indicate lower values per peptide bond. The quantum yield is independent of the presence of oxygen. The primary process has a temperature coefficient of about unity, which is typical of many photochemical nonchain reactions. A second step, possibly involving a reaction with solvent, is highly temperature-dependent in the one case studied (egg albumin). In the presence of oxygen subsequent degradation is different from that in its absence and leads to the formation of colored and odoriferous products. Accompanying denaturation-inactivation is a disruption of the molecule which leads to a decrease in average molecular weight and a tendency for agglomeration of products which, at the isoelectric point or in the presence of salts, may result in precipitation. Other manifestations are changes in solution viscosity, diffusion coefficient, electrophoretic mobility, and dialyzability. Other evidence that the molecule has undergone deep-seated changes rests in observed alterations in refraction of light, optical rotation, refraction, absorption spectra, surface tension,  $pH$ , gold sol reaction, and liberation of sulfhydryl groups, ammonia, and aldehydes. The precise meaning of these changes is still obscure; they result from degradation with the formation of mixtures of molecules of unknown structure. For a better interpretation, there is needed a quantitative correlation of denaturation-inactivation with the physical and chemical modifications observed. The site of action of absorbed quanta is in the neighborhood of the chromophore but may be three atoms away as in rupture of peptide bonds. The chromophore itself may, as tyrosyl, undergo degradation. It is not known whether only splitting of a peptide bond will result in denaturation or whether photooxidation of a chromophore will also suffice. Insoluble proteins may be solubilized by photodepolymerization and loss of cross linkages. Some irradiated substances remain antigens, others do not. The degradation products sometimes have altered physiological activity, as drugs or as substrates for enzymolysis. Proteins in solutions containing alcohol, acetone, or salts are more easily denatured by light indicating the less hydrated protein molecule is more susceptible to denaturation. Denaturation by light arises by a mechanism distinctly different from that induced by any of the other known agents. It is irreversible.

#### IV. Action of Radiation on Enzymes, Hormones, Viruses, and Bacteriophages

In 1879 Downes and Blunt reported that zymase (invertase) was destroyed by sunlight (104). Amylase was first inactivated by Green in 1897 (148). First order rates of destruction were observed for rennin (180,301a), pepsin, and papain (180) more recently. That  $pH$  of the media during irradiation is important was recognized by Pincussen *et al.* for malt amylase (261), urease (271), catalase (278), and pepsin (279). They believed that maximum destruction occurs at the  $pH$  of optimum activity of the enzyme. Further points of historical interest may be found in a review by Schomer (302).

##### A. QUALITATIVE STUDIES WITH ENZYMES, HORMONES, VIRUSES, AND BACTERIOPHAGES

Among the large number of experiments cited, it can be seen that in most of the studies little use has been made of quantitative methods. This was natural since the application of precise photochemical technique to substances of unknown purity could hardly be expected to yield results interpretable in a precise way. An exception to this principle was the work of Warburg *et al.* with the respiratory enzymes. Here the success of the method rested partly on assumption of a constant quantum yield at all wavelengths used and partly on nondestruction of the proteins at those wavelengths (355). However, Warburg's work did not involve UV light of such short wavelengths as to cause protein denaturation, a generality at wavelengths less than 3000 Å. Nevertheless, the use of monochromatic light instead of "shot gun radiation" can serve to show a wavelength dependence of inactivation rates if energies are carefully measured. The time has now arrived to attempt to correlate protein structure with photochemical behavior since many proteins have been crystallized, their molecular weights determined, and their chemical role, such as enzyme specificity, partially established. Loss of ability to act as an enzyme, a hormone, or a virus can now serve as a measuring stick against which physical and chemical changes can be compared. The number of times a protein molecule is hit on the average up to the time of change in chemical identity can now be "counted."

Too often in the earlier work no attempt was made to control  $pH$ , evaporation, and temperature, or to insure adequate stirring during

TABLE VII  
ENZYMES IRRADIATED WITH ULTRAVIOLET LIGHT

Enzyme	Effects and remarks
Aldehydease (milk) ....	Maximum rate of destruction at pH optimum, 7.35 (276).
Amylase <sup>a</sup>	
(Aspergillus) .....	Destruction spectrum studied (78).
(Malt) .....	Inactivation (9). Inactivation more rapid than with yeast invertase (79). Temperature coefficient of inactivation 1.3 between 20 and 30° (218).
(Pancreatic) .....	Inactivation (9).
(Sweet potato) .....	Inactivation rate reduced by presence of vitamin C (139).
(Yeast) .....	Inactivation (242,243).
Carboxypeptidase .....	Quantum yield determined at 2537 Å. (224)
Catalase .....	Inactivation (8,241). Inactivation rate greater in alkaline than in neutral or acid solutions (354). Inactivation by visible light in presence of O <sub>2</sub> (27, 184,368).
(Blood) .....	Inactivation (278).
(Liver) .....	No inactivation by light >3000 Å. (331).
Chymotrypsin .....	Inactivation by first order kinetics; quantum yield determined at 2537 Å. (120).
Diastase (malt) .....	Inactivation (148). Reciprocity law holds in inactivation (181). Inactivation rate dependent on pH (261). Protected by 6 M phosphate (261). Greatest effect at optimum pH by monomolecular reaction (262). Inactivation rate reduced by presence of salts (265,266,267)
(Pancreas) .....	Sensitivity toward light increases with purification (264). Partial reactivation by addition of active enzyme preparation; not true with heat-inactivated enzyme (274).
(Pancrease, dog) .....	Inactive form (NaCl-free) more stable than active form (with NaCl) (343).
(Pancreatin) .....	First order inactivation (346).
(Saliva) .....	Reciprocity law holds in inactivation (181).
(Snail) .....	More sensitive to destruction when exposed with NaCl than in inactive, dialyzed form (343)
(Taka) .....	Inactivation <i>in vitro</i> , not <i>in vivo</i> (129). Inactivation rate greater than for malt diastase (263). Greatest rate of inactivation at pH optimum (274).
Emulsion .....	Inactivation (8). Differential rate of inactivation toward $\beta$ -D-glucoside and $\alpha$ -D-mannoside (159) (probably a mixture of enzymes, in light of recent considerations, 227).
Enterokinase .....	Partially heat-denatured enzyme undergoes further inactivation by irradiation of 2811 Å. (255).
Ficin	
(Crystalline) .....	Inactivation by first order kinetics (229).

TABLE VII (*continued*)

Enzyme	Effects and remarks
Invertase .....	Irradiated solutions lose residual activity on standing (104). Inactivation (8,185). Ca ion must be present for inactivation (200). Inactivation rate greater with increasing purity (334).
(Difco) .....	Inactivation <i>in vitro</i> but not <i>in vivo</i> (128).
(Yeast) .....	Inactivation in presence of O <sub>2</sub> (104). Degree of inactivation slightly dependent on pH (143). Absorption not measurably changed with inactivation; destruction may involve tryptophan (144).
Laccase .....	Inactivation (9).
Lecithinase (Taka) ....	At pH 6.6 inactivation rate greater than for phosphatase (275,276).
Lipase (guinea pigs) ...	Inactivation at acid pH; almost nil at alkaline pH (270).
(Rabbit serum) .....	Inactivation at acid pH; almost nil at alkaline pH (270).
Lipase .....	Inactivation (337).
Luciferase ( <i>Cypridina</i> ) ..	Inactivation by first order kinetics at <3000 Å.; at >3000 Å. photosensitized by Bismarck brown; has typical protein absorption spectrum (77).
Lysozyme (egg) .....	Inactivation (187).
Maltase .....	Inactivation (112).
Papain .....	Inactivation (192).
Pepsin .....	Inactivation (9). Maximum denaturation rate at pH 1.9 with albumin as substrate (93). Inactivation at first order rate; maximum rate at pH 1.15 (180). Claimed that absorption spectrum coincides with destruction spectrum (134). Inactivation rate reduced in presence of vitamin C (139). Quantum yield at 2537 Å. determined as function of pH (226). Maximum denaturation rate at pH 1.4 (279). Maximum denaturation rate at pH 2.0 with hemoglobin as substrate (250).
Peroxidase .....	Inactivation by visible light in presence of O <sub>2</sub> (27).
(Horse-radish) .....	Inactivation rate not a function of pH (269).
(Milk) .....	Inactivation in milk (287).
Phosphatase (liver) ....	Inactivation rate reduced by presence of vitamin C (139).
(Serum) .....	Inactivation due to direct destruction of protein carrier (10).
Rennin .....	Inactivation (8,12).
Succinic dehydrogenase (horse muscle) .....	Inactivation (277).
Sucrase .....	Inactivation rate not a marked function of pH; apparent activation at beginning irradiation may result from photooxidation of inhibitory substances (142,145,146).

TABLE VII (concluded)

Enzyme	Effects and remarks
Trypsin .....	Inactivation (69,192). Partially heat-denatured enzyme undergoes further inactivation by irradiation at 2800 Å. (255). Inactivation by first order kinetics; quantum yields determined at 2537 and 2804 Å. (349). Quantum yields at several wavelengths determined and several substrates used (352).
Tyrosinase .....	Inactivation (8,268,269).
(Dolichos) .....	Temporary increase in activity claimed (245).
Urease, soybean .....	Inactivation (183,271,272,340).
(Amorphous) .....	Inactivation; claim that absorption and destruction spectra coincide (194).
(Crystalline) .....	Inactivation; found that absorption spectrum and destruction spectra do not coincide (198). Inactivation by one hit process with 1/1 loss in activity and change in position in sedimentation constant, i.e., all-or-none denaturation (227).

\* An amylase hydrolyzes polysaccharides to glucose. If found in the pancreatic juice, saliva, or plants it is often called amylopsin, ptyalin, or diastase, respectively.

exposure. Consequently a large fraction of the early work will need to be repeated with pure proteins under controlled conditions. The only significant generality forthcoming from many of these studies is the fact that UV light will cause inactivation or denaturation and ultimately degradation (323).

The relation of UV light to enzymes may be divided into its use in studying enzyme structure via absorption spectra and the effects of the absorbed energy on structure (302). Only recently has the first use brought forth more than obvious answers. The findings of Neuberg and Crammer, Sizer, and Darby concerning the behavior of "bound" tyrosine may be cited. The second use has involved many physical methods which have yielded results difficult to interpret and a paucity of chemical measurements with poor to unsatisfactory correlations of light absorbed with chemical reaction. Future chemical investigations will doubtless be associated with preliminary determinations of quantum yields for denaturations and for splitting of bonds, as begun by Uber and his students and by Rideal and Mitchell, and with the use of model substances. The use of model substances, i.e., peptides, has hinged partially upon the development of sensitive chemical methods for determining small amounts of split products. These are now available. Early



TABLE VIII

## HORMONES, VIRUSES, AND BACTERIOPHAGES IRRADIATED WITH ULTRAVIOLET LIGHT

Protein	Effects and remarks
Bacteriophage ( <i>Staphylococcus aureus</i> )	....High sensitivity near 2650 A. (135).
Dysentery phages	.....Inactivation by one hit process at 2537 A. (217).
Encephalitis virus	.....Loss of activity (219).
Equine encephalomyelitis virus	.....Loss of activity by first order rate (341).
Herpes virus	.....Loss of activity (341).
Influenza virus	.....Maximum sensitivity at 2650 A.; similar to vaccine virus and bacteriophage (172). Loss of activity (295,362).
Insulin	.....62% total N <sub>2</sub> liberated as NH <sub>3</sub> following inactivation (5,6). Loss of activity under air or N <sub>2</sub> (67, 75, 100, 110,306). Tyrosine is split out of monolayers (73). Forms products with hyperglycemic activity (110). Partial reversal of lost activity by action of H <sub>2</sub> O <sub>2</sub> with cysteine (125). Irradiation reported to cause increase in activity followed by decrease (183,353). Liberates sulfhydryl groups (191). Light >3000 A. ineffective; most effective region between 2400 and 2725 A. (195). H <sub>2</sub> S, NH <sub>3</sub> , probably aldehydes liberated (195). Hydroxyl ion reported to have protective action (197). Quantum yield determined (223).
Poliomyelitis virus (SK-mosaic)	.....Inactivated virus usable for immunization (220).
Roux sarcoma virus	....Slight maximum in sensitivity at 2600 A. with greatly increased sensitivity at shorter wavelengths (333).
Secretin	.....Loss of activity accompanied by liberation of 97% total N <sub>2</sub> as NH <sub>3</sub> (6).
Tobacco mosaic virus	..Slight maximum in sensitivity at 2600 A. with greatly increased sensitivity at shorter wavelengths (171). Quantum yield for inactivation determined at 2537 A. (225). First order inactivation (282). Loss of activity but still crystallizable (327).
Tomato bushy stunt virus	.....Loss of activity but substance can still be crystallized and retains serological activity (32).
Vaccinia virus	.....High sensitivity near 2650 A. (291).

reports concerned with the action of UV light on enzymes are summarized in Table VII. A more complete history may be found elsewhere (302).

At present it is not known whether the breakage of a single bond anywhere can result in the loss of biological activity or whether certain specific bonds must absorb light in the inactivation process.

It is also not known generally whether the breakage of any bond will result in complete denaturation with a simultaneous loss in activity, or whether inactivation can occur without denaturation. A complete story will in part await a knowledge of the photochemistry of the groups essential for biological activity. Recent knowledge of these groups has been summarized by Olcott and Fraenkel-Conrat (254). Those involved include phenol (chymotrypsin, papain, pepsin, insulin (332), tobacco mosaic virus), sulfhydryl (papain), carboxyl (lysozyme, pepsin, insulin), disulfide (insulin), and amide or guanidyl (lysozyme). For these groupings there is a paucity of quantitative data concerning photochemical behavior in solution.

Hormones, viruses, and bacteriophages which have been irradiated are cited in Table VIII.

## B. QUANTITATIVE STUDIES WITH ENZYMES AND VIRUSES

### 1. *Pepsin*

Pepsin has been inactivated as both crude (93,180) and crystallized (132,133,226,250) preparations. All agree that the rate is first order. Burge observed that light at less than 3130 Å. was effective (66). The classical photochemical study of Northrop helped to establish the fact that pepsin activity during irradiation was proportional to loss of solution protein, *i.e.*, the activity per milligram protein nitrogen in solution remained constant. The reason for this was that as fast as denatured protein was formed it was digested by the remaining enzyme.

Gates sought to determine the relation existing between radiant energy absorbed by pepsin and the amount of inactivation. Loss of activity was determined by hemoglobin proteolysis. Results obtained at various wavelengths plotted against incident energy, at pH 2.06 (25–27°) are shown in Figure 15. The one quantum relationship is seen to hold well. This work of Gates was written up by Crozier and Oster after his death. The authors present the data in an attempt to show a direct correspondence between the inactivation spectrum and the absorption spectrum.

The meaning of an action spectrum can be shown as follows: If absorption is small, say less than 10%, we may write, for constant concentration (*c*) and path length (*l*):

$$I_{ab} = \epsilon I_0 c l = k I_0$$

where  $k$  is a constant for a given wavelength. Thus the product of an absorption coefficient,  $k$ , and the incident intensity provides a measure of the rate of absorption of energy by the absorbing substance. From the above  $E_{ab} = E_0 k$ , where  $E_{ab}$  and  $E_0$  are the absorbed and incident energy, respectively. In order to compare the effects at different wavelengths, one must compare the number of absorbed quanta rather than the absorbed energies, since the former determined the extent of the photochemical reaction. The number of quanta,  $N$ , is related to  $E_{ab}$  by  $N = E_{ab}/h\nu = E_{ab} \lambda/hC$ , or  $N$  is proportional to  $E_0 k \lambda$ . Making the assumption that the same number of quanta is required to produce the given inactivation or response (i.e., a constant quantum yield) regardless of wavelength the result is  $1/E_0 \propto k\lambda$ . Thus to obtain an action spectrum one needs to know the incident energy,  $E_0$ , for

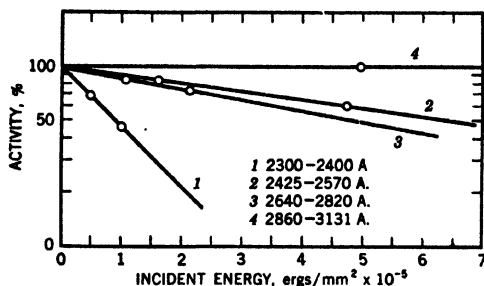


Fig. 15. Inactivation of pepsin at different parts of the spectrum (133).

a number of wavelengths, required to produce a given degree of inactivation and to plot  $1/E_0$  against wavelength. The greater the response for a given incident number of quanta, the greater is the absorption of quanta by the molecule. The above conditions are fulfilled in some photobiological processes to demonstrate accurate agreement between action and absorption spectra, but not in the majority (50). It also holds for the Warburg *Atmungsferment* (355).

Even casual inspection of the result shows that agreement between inactivation and absorption spectra is poor. Landen (198) believes Gates' conclusion to be contrary to the data presented. Using the point wavelengths of the bands (Fig. 15) Landen recalculated the data of Gates and obtained 0.0014, 0.00034, 0.00045, and  $\sim 0.0$  at 2357, 2509, 2719, and 2930 Å. for the quantum yields (Fig. 16). In view of the multiplicity of the kinds of absorbing linkages and chromophores in proteins, all of which probably photolyze with different efficiencies, a constant quantum yield for inacti-

vation with change in wavelength is not anticipated. An action spectrum cannot be made to coincide with an absorption spectrum unless the quantum yield is constant for the wavelength region involved. Working at 8 and 22°C., a temperature coefficient of 1.02 was found by Gates at 2537 Å. for the inactivation of pepsin at pH 2.1 (134). Such a value is consistent with typical photochemical behavior of smaller molecules.

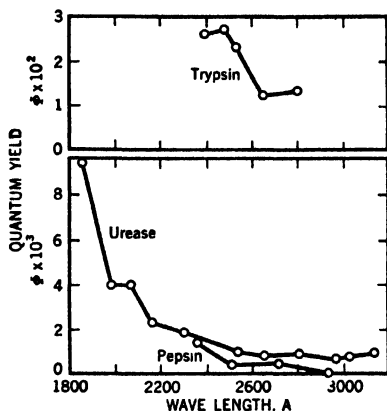


Fig. 16. Quantum yields,  $\phi$ , plotted against wavelength for trypsin, pepsin, and urease (221).

Using a mercury arc, Collier and Wasteneys irradiated crude pepsin solutions at 2000–3130 Å. as a function of pH with egg albumen as a substrate. A plot of the velocity of destruction, as  $k$  of the first order deactivation equation, against pH is shown in Figure 17 from their paper. The curve shows a pronounced maximum at pH 1.9, a minimum at pH 4.8, and a rapid increase as pH 7 is approached. Above pH 7 pepsin undergoes spontaneous deactivation (251). Only a small fraction of  $k$  is attributable to changes in light absorption (226), therefore the variation of  $k$  with pH is actually a variation of quantum yield.

A possible partial interpretation of the pH dependence of quantum yield is as follows: At pH values of maximum stability (5.28 for heat and 4.25 for light) the number of salt bonds is a maximum. With the addition of alkali the number of un-ionized carboxyl groups

decreases, the number of hydrogen bonds decreases, and a strong repulsive force of ionized carboxyl groups becomes operative, which tends to stretch the molecule. (The number of basic groups is relatively small in pepsin.) This is analogous to the titration of polymethacrylic acid, to which a similar statement applies (188). In

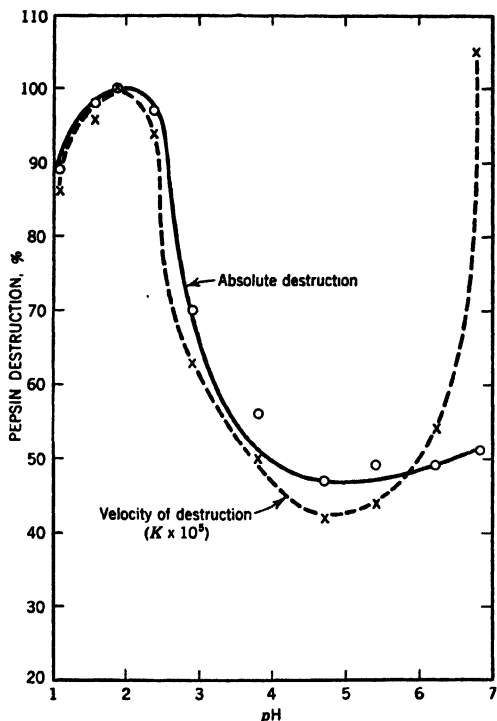


Fig. 17. Relation between pH and rate of destruction of pepsin by ultraviolet light (93).

this strained, ionized condition less activation energy is required for photochemical breakdown. (For thermal denaturation, incidentally, the molecule must first acquire energy for the ionization of five ionizable, probably cystenyl amino, groups as postulated for pepsin by Steinhardt (329)). This picture is similar to that of Eyring and Stearn for heat denaturation of pepsin (114). The photochemical

step probably consists of breakage of an  $\text{—S—S—}$  or a  $\text{—CONH—}$  bond, which, because of a decrease in tendency for the resulting radicals to recombine resulting from the ionic repulsion of the large negatively charged radicals at higher  $p\text{H}$ , leads to a higher quantum yield. It is probable that a lower quantum yield would be found by irradiation under pressures of a few thousand pounds per square inch. The increasing failure of radical split products to recombine with increasing  $p\text{H}$  means a decrease in an intramolecular Franck-Rabinowitch effect (see below). A similar mechanistic explanation below  $p\text{H}$  4.25 is more difficult and none is offered. It appears from the results of Goulding, Borsook, and Wasteneys (147) that pepsin is unionized in the range 1 to 4.5 (see 164, however). There is doubt as to whether pepsin has an isoelectric point at  $p\text{H}$   $>1$  although values ranging from 1 to 2 have been reported (251). The  $\Phi$  *vs.*  $p\text{H}$  curve in this region is similar to the  $p\text{H}$  *vs.* rate of activity curve for pepsin (93). It is hoped that work with polydi-basic amino acids as model substance for quantum yield studies will help in understanding these results.

The quantum yield ( $p\text{H}$  2.01) was found to be the same with either hemoglobin or casein as a substrate, namely,  $2.5 \times 10^{-3}$ , at 2537 Å. Urea had no effect on the quantum yield. It has been concluded that inactivation can occur by the absorption of a quantum by any aromatic group in pepsin (226).

## 2. Trypsin

The inactivation of trypsin by UV light is reduced by iodides of the alkalis or alkaline earths (255,273). Calvin reported a decrease in trypsin activity during irradiation by a mercury vapor lamp (69). An impure (1/110) trypsin preparation was also irradiated by Kauffman and Urbain. Their findings are difficult to interpret, however, inasmuch as their data are given in terms of "energy in watts per square centimeter." They agree however that trypsin and papain are destroyed by UV light (192).

Quantum yields have been reported for trypsin as 0.017 and 0.016 at 2537 and 2804 Å., respectively, based on its loss of ability to release carboxyl groups in  $\alpha$ -benzoyl-L-argininamide hydrochloride. In these experiments the concentration of active trypsin decreased exponentially with time of irradiation at constant intensity (since the primary products and reactants absorb almost equally)

indicating that a molecule of enzyme must be inactivated by a single quantum. On the average, however, only one quantum out of sixty is effective. Since most of these quanta are absorbed by the aromatic amino acid residues, of which there are probably about twenty in the trypsin molecule, the question arises as to whether some particular aromatic residue must absorb a quantum to produce inactivation of the molecule, with a yield of around one-third for that residue, or whether any aromatic group may do so, but with a yield of about one-sixtieth. The latter is probably correct (226).

Verbrugge (351) was interested in whether or not the method of assay of inactivated enzyme or the nature of the substrate influenced the calculations of quantum yields at various wavelengths. Much of his attention was centered about the hypothesis of several active centers per molecule. He reasoned that if these centers are independent of each other all must be inactivated by absorption of quanta in order to produce complete inactivation of each molecule. In other words, "the loss in activity as measured experimentally will be directly proportional to the true extent of the inactivation only if the loss of activity at one locus does not affect the activity of remaining active centers." This argument has at least two major weaknesses. First, no one has as yet demonstrated that a proteolytic enzyme has more than one active center or that it has an active center which may act without the molecule being intact. Secondly, experiments of Clark (90) on solubility loss of albumin during irradiation indicate that denaturation of the entire molecule takes place by a one hit process (Verbrugge makes the statement that enzymes can lose their activity without becoming denatured since Northrop (250) denatured pepsin by light without finding denatured protein; pepsin digests denatured protein, as Northrop points out, so that of course no denatured protein was found), and McLaren and Sheppard (227) have found a one-to-one relationship in loss of activity of urease and loss of identity in the ultracentrifuge sedimentation diagram, showing all-or-none behavior in destruction by UV light. In other words, granting multiple active centers for enzyme molecules, it would seem that a single quantum is capable of denaturing the entire molecule. The same difficulty obtains for the proposition that "a similarity in wavelength dependence in quantum yields as measured by the various methods would tend to support the hypothesis that the active centers are

alike in their action and, therefore, probably alike in their chemical constitution." The results of Verbrugge's study are reproduced in Table IX. Verbrugge found that at 2804 Å. the quantum yield is only half the value at 2537 Å. with benzoylargininamide as substrate, whereas Uber and McLaren found the same yield at the two wavelengths. He attributes this to a lower extinction coefficient at 2537 Å. for his trypsin preparation. Uber and McLaren, as well as Kunitz (196), give about the same molecular extinction coefficient at 2537 Å. for trypsin. Verbrugge generously furnished this author with a sample of his trypsin and in this laboratory it was found that his trypsin had a molecular extinction coefficient of 2.2 instead of 1.6

TABLE IX  
QUANTUM YIELDS FOR TRYPSIN INACTIVATION (351)

Substrate	Method	Wave-length, Å.	Av. quantum yield
Benzoylargininamide	Grassman-Heyde titration	2399	0.025
		2537	0.024
		2650	0.012
		2804	0.013
Hemoglobin	Anson colorimetric	2399	0.087
		2537	0.103
		2650	0.053
		2804	0.048
Hemoglobin	Formol titration	2399	0.027
		2483	0.027
		2537	0.040
		2804	0.015
Casein	Colorimetric	2537	0.019
		2804	0.011
	Grassman-Heyde titration	2537	0.026
		2804	0.012

as reported previously at 2537 Å. Since Verbrugge computed the amount of light absorbed in his radiation experiments from his absorption curve (352) the apparent differences in quantum yields at 2804 and 2537 Å. offered by him are probably less pronounced than reported. Nevertheless, it appears that the data of Table IX activation energies, heats of denaturation, and quantum yields.



with decreasing wavelength has been demonstrated. (With hemoglobin a higher  $\Phi$  was found at 2537 than at 2483 and 2804 Å. This is also explained by the error in absorption spectrum at 2537 Å. The yields at the three wavelengths are probably nearly the same.) There can be little doubt, however, that the method of determination may result in variations, within a factor of two, in the quantum yield calculated, since the colorimetric determination with hemoglobin is definitely higher than with a titration procedure for the same substrate.

From the high quantum yields with hemoglobin as substrate, Verbrugge reasons that, if one assumes that the several active groups in the trypsin molecule simultaneously attack the many susceptible bonds in a hemoglobin molecule, a fairly large number of split products will result. If during irradiation some of the active centers are destroyed, hydrolysis of hemoglobin by such a molecule will result in larger split fragments, fewer of which will appear in the filtrate (used for the colorimetric analysis) resulting in an apparently higher quantum yield. Both the colorimetric and the titration method gave identical yields with casein as a substrate, however. It is of interest to note that Verbrugge's idea of several active centres on an enzyme molecule results from his interpretation of Herriott's work on the acetylation of pepsin (163,165). Herriott does not see how this conclusion could have been reached (162).

### 3. *Chymotrypsin*

The typical exponential decline in activity of chymotrypsin with irradiation (120) at pH 4.65 by  $\lambda$  2537 Å. is shown in Figure 18. Chymotrypsin has at least a duospecificity toward peptides, *i.e.*, it is both an amino peptidase and a carbonyl proteinase (126). It also has a limited esterase activity (304). The question arises as to whether or not one kind of functional group(s) is responsible for all these activities, or whether a single species of active center is responsible for the hydrolysis of each of the substrate types. One might suppose that the preferential absorption and action of UV light could result in a preferential rate of breakdown of the several kinds of active centers if such exist. On the other hand, if an all-or-none inactivation takes place, as seems to be the case with urease, a selective rate of breakdown of one functional group would

not show up because of a simultaneous complete denaturation of the entire molecule. Thus experiments designed to test this hypothesis, using substrates toward which chymotrypsin has different specificities, would be useful only if they did show this preferential action. If they did not, one could not automatically conclude that only one kind of functional group is present. The same reasoning applies to the use of heat denaturation (304) to this problem. The quantum yield is the same under nitrogen or oxygen at  $pH$  3, although the

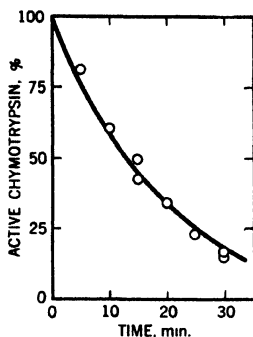


Fig. 18. Loss in activity of chymotrypsin with time of irradiation (120).

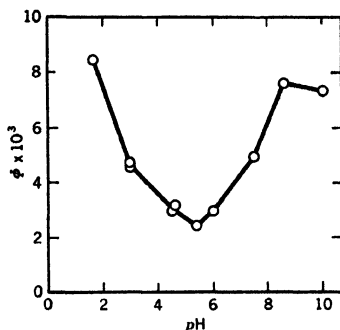


Fig. 19. Variation of quantum yield with  $pH$  for inactivation of chymotrypsin (119).

absorption spectra of the inactivated enzymes is a function of the atmospheric conditions under which it was irradiated. This is similar to Bernhart's findings (44). The quantum yield is also a sensitive function of  $pH$ , having a minimum near the  $pH$  of maximum thermal stability of the enzyme (Fig. 19) (119).

The explanation for the increase in quantum yield at high and low  $pH$  values is similar to that offered for pepsin: At high and low hydrogen ion activities either a preponderance of ionized acid or basic groups result in internal repulsion. The internal repulsion may tend to reduce a Franck-Rabinowitch effect.

#### 4. Urease

Crude urease from soybean has been irradiated by Pincussen and associates (271,272). Crystalline urease (after Sumner) has been studied qualitatively by Tauber (340), and quantitatively by Lan-

den (198). A presumably pure but noncrystalline preparation was also investigated by Kubowitz and Haas (194). Tauber found that only in the presence of eosin did sunlight have an effect on urease. UV light had an inhibitory effect upon activity inversely proportional to the distance from the radiation source. The inhibitory action was enhanced by the presence of eosin. Pincussen observed that the enzyme was destroyed by irradiation and that boiled urease has a reactivating effect (272). Pillemer *et al.* were also interested in the influence of various reducing agents on irradiated urease (260). This was of value partly because Hellerman, Perkins, and Clark subjected urease to oxidation and reduction and demonstrated the reversibility of the reaction (151). Pillemer *et al.* showed that urease which had been oxidized by aeration or iodine may be largely reactivated by hydrogen sulfite or potassium cyanide. After exposure to UV light (carbon arc) the enzyme was permanently destroyed; it could not be reactivated by reducing agents or tissue extracts. The course of the inactivation was not influenced by irradiation under nitrogen, or in the presence of potassium cyanide or hydrogen sulfide.

At Warburg's suggestion Kubowitz and Haas irradiated urease with the aid of a monochrometer and mercury arc and metallic spark sources. A bolometer was used to measure intensities at the wavelengths studied. They assume a constant quantum yield for the inactivation of urease in the UV region of the spectrum and by so doing were able to show a correspondence between the inactivation and the absorption spectra with good agreement except at 2540 Å.

At Uber's suggestion Landen repeated this work with urease using crystalline enzyme. As Landen pointed out, this was especially worth while since Gates also claimed a direct correspondence between absorption and action spectra with pepsin (see above). For this study a crystal quartz monochrometer was used in conjunction with a capillary mercury vapor lamp or zinc or aluminum spark sources. Intensity measurements were performed with a vacuum thermopile (Eppley). During all irradiations the solutions, of small volume exposed to the air, were stirred with a quartz rod. Except for slight evaporation losses these experiments were as accurate as one could normally expect. The buffers used were the same as those of Kubowitz and Haas (pH 6.5). Absorption measure-

ments were acquired by both a Spekker photometer and a photocell-direct current amplifier. Landen's results are in agreement with those of Kubowitz and Haas below about 2400 Å. At longer wavelengths Landen's results are higher.

After the work of Kubowitz and Haas the molecular weight of urease, 483,000, was published. Using their data Landen calculated a yield of 0.003 molecules per quantum throughout the spectral range used (1960–3660 Å.) except at 2540 Å. Landen in turn studied urease to check the value at 2540 Å. and to determine whether or not the quantum yield is really constant. His doubt was centered in the fact that Gates' data for pepsin were not consistent with Gates' conclusion, namely, that the destruction spectrum and action spectrum coincide. The values in Figure 16 were obtained by use of the expression for the quantum yield:

$$\Phi = \frac{NV(c_0 - c)}{\frac{I_0 l}{hC/\lambda} T \frac{\epsilon c_a}{\epsilon c_0 + k_B} [1 - 10^{-(\epsilon c_0 + k_B)l}]}$$

(Note that the molecular extinction coefficient,  $\epsilon$ , was left out in the numerator of the ratio  $\epsilon c_a / \epsilon c_0 + k_B$  in the original paper.) Here  $N$  is Avogadro's number,  $V$  is the irradiated volume in liters,  $c_0$  is the original, and  $c$  the final concentration of the active urease in moles per liter,  $I_0$  is the incident energy in ergs per second,  $t$  is the time in seconds,  $C$  is the velocity of light in centimeters per second,  $T$  is the transmission of the window of the quartz cell at the wavelength of the experiment,  $c_a$  is the average concentration of the active urease (formed by integrating over the value of the dose),  $k_B$  is the extinction coefficient of the buffer solution, and  $l$  is the thickness of the solution in centimeters. The term  $\epsilon c_a / (\epsilon c_0 + k_B)$  gave the fraction of the total radiant energy absorbed by the active urease during a given trial. Since the absorption coefficient of urease,  $\epsilon$ , did not change until more than 85% was inactivated, the original concentration was used for calculating the total absorbed energy. These data show that the quantum yield is fairly constant from 3130 to 2537 Å; the average value is about 0.0008. At shorter wavelengths the yield climbs to 0.00938 at 1860 Å. It is unfortunate that these results and those of Kubowitz and Haas are so incompatible; the yields of Landen vary with wavelength and give values from one-

fourth to three times the value 0.003 found by Kubowitz and Haas. Obviously this entire study must be repeated by others. The difference may rest partially in differences in absorption spectra, in methods of assay used, and in irradiation technique. Kubowitz and Haas irradiated solutions so dilute as to absorb from 1 to 5% of the incident irradiation, whereas a large fraction of the incident intensity was absorbed in the experiments of Landen.

### 5. *Equine Encephalomyelitis Virus*

Equine encephalomyelitis virus protein (eastern strain) has been studied by Taylor *et al.* (341). Measurements of the absorption spectra of this substance in purified solution reveal qualitatively that the curve is similar to that of nucleic acid even though the virus contains only 4.4% of it. Solutions containing pure nucleic acid in concentration comparable to that of the intact virus protein gave much smaller values for the extinction coefficient so that no quantitative relation with the virus was evident. This discrepancy, the reviewer believes, was probably in part due to light scattering by the intact virus, the molecular weight for which is doubtless quite high. The same kind of discrepancy has been noted for tobacco mosaic virus (see below). A study of the rate of inactivation of infectivity associated with the protein by monochromatic light (2537 Å.) revealed a linear relationship between the logarithm of the survival ratio and the time of irradiation for dilute solutions. With more concentrated solutions the logarithmic relationship did not apply; the decrease in rate was found to be partly due to increase in absorption during irradiation. This phenomenon is not observed with tobacco mosaic virus, incidentally (see below).

The rate of inactivation of infectivity (at 2537 Å.) was found to be of the same order of magnitude as that for the bacterium *Serratia marcescens* under similar conditions. There is no *a priori* reason for deducing more than a coincidental comparison, however, since the degree of light scattering, the site of absorption, the presence of dissolved gases, etc. can be contributory factors to both experiments and in unknown relative amounts.

Inactivation of the virus did not affect the sedimentation pattern or constant of the virus (see above statements on studies with the ultracentrifuge). This is surprising since any changes in UV absorp-

tion spectra indicate rather drastic changes in chemical composition at 2537 Å. That these changes did not result in changes of size or shape of the individual particle as indicated by constancy of the sedimentation constant is noteworthy.

### 6. Tobacco Mosaic Virus

Shortly after isolation of tobacco mosaic virus in pure form, Stanley reported qualitative experiments on the inactivation of the crystalline protein (327). It was found that treatment with hydrogen peroxide, formaldehyde, nitrous acid, or UV light produces inactive proteins, which, although slightly altered, retained certain chemical and serological properties characteristic of the virus protein. (The opalescence observed in irradiated solutions by Stanley—a full mercury arc was employed—was probably partially due to heat denaturation since recent work has shown that irradiation of cool solutions with light of 2537 Å. produces inactivation without opalescence (225). If the temperature of the solution climbs to 37° during irradiation opalescence and a faint yellow fluorescence are produced.) The inactive native protein was found to be still soluble but tended to be more easily “denatured” than the active material. The optical rotation of solutions of protein previously irradiated was practically unchanged. The same was true of the isoelectric point. A study of UV-inactivated virus in the ultracentrifuge disclosed that no marked change in molecular weight occurred. Crystals may be prepared of native and UV-inactivated virus. The sera precipitin reaction is also unchanged following irradiation of the virus (327). Following inactivation (2537 Å.) no changes in UV absorption spectrum or viscosity of solutions have been found. Electron micrographs before and after revealed no change in particle size or shape (225). This remarkable physical stability may be contrasted with the behavior of urease or of the other pure enzymes studied thus far. Under conditions which completely inactivate the virus, derived nucleic acid in solution undergoes little if any change in viscosity with irradiation. Apparently nucleic acids lend a stabilizing framework to the virus.

A high sensitivity has been reported for influenza virus, vaccinia virus, and bacteriophage at 2650 Å. with a decrease in sensitivity at longer and shorter wavelengths. By contrast, typical tobacco mosaic viruses and Roux sarcoma show but a slight maximum sensi-

tivity at 2600 Å. with greatly increased sensitivity at shorter wavelengths. The former contain mostly desoxyribonucleic acid and the latter ribonucleic acid. Ribonucleic acid is probably less sensitive to 2537 Å. than is desoxyribonucleic acid (170).

### C. SUMMARY

It has been known for many years that radiation inactivates enzymes, hormones, bacteriophages, and viruses. There is no substantiated exception with wavelengths shorter than about 3000 Å. Longer wavelengths are active in the presence of photosensitizers. Of the large number investigated only six have been studied at all methodically. Inactivation is always "monomolecular." So far, chymotrypsin and pepsin have been shown to have a sensitivity to radiation which is pH dependent. Pepsin, trypsin, and urease show increasing quantum yields as wavelength is reduced. Hence action spectra do not coincide with absorption spectra. At present there is a controversy about whether or not the quantum yield is dependent on the substrate used for analysis. Physical evidence is strongly indicative that no such dependence will really be found. The apparent all-or-none nature of UV denaturation-inactivation prohibits this. Further data of greater accuracy will serve to clarify this issue. Quantum yields for enzymes are, roughly speaking, smaller the larger the molecular weight but no exact proportionality is extant. With enzymes irradiation brings about the same physical and chemical changes noted for proteins generally. Tobacco mosaic virus is a special case; inactivation is possible without any observable changes in size, shape, or chemistry. After inactivation it is still crystallizable. Nucleic acid is depolymerized by UV light, but combined with the virus protein an exceedingly stable structure is produced and inactivation is a most subtle process. Inactivation of this virus perhaps occurs through the breakage of a single bond and without denaturation. An obvious experiment is to look for light-induced mutations in the virus and then study the chemical composition of the new strain, if produced. It is hoped that further work with the enzymes and especially the viruses will lead to an understanding of light-induced mutations in genes.

## V. Quantum Yields of Enzymes, Proteins, and Viruses

The known quantum yields for proteins are summarized in Table X.

As has been shown, the hydrogen ion concentration is of importance in quantum yield studies and must be specified in any such tabulation. It is also apparent from previous discussion that the method of assay used in following the inactivation may also influence the numerical values. Neither of these variables seems to be responsible for a variation of more than a factor of two or three in the quantum yield; the value found in the region of the pH of maximum thermal stability is probably a fundamental characteristic of the protein.

It was suggested that the quantum yields for proteins may be inversely proportional to their molecular weights, by Uber, at a time when few yields were known (348). The quantum yields of the proteins (Table X) do not bear such a close relationship to molecular weight nor are they proportional to the square root, cube root, or fractional power of the respective molecular weights. Actually a plot of  $1/\Phi$  against the log molecular weight gives some semblance to a straight line. Such a plot may have value in helping to predict the order of magnitude of a quantum yield for a protein. Uber's idea suggests that the number of quanta required to inactivate a molecule might be associated with the number of chromophores. This association is further indicated by the calculation of Rideal and Mitchell regarding the quantum yield per chromophore in ovalbumin as a monolayer. As was described above, these authors found a yield of nearly unity for the breakage of peptide linkages adjacent to tyrosine residues. In Table X the approximate number of aromatic residues per molecule for the proteins studied are tabulated. Only at the extremes of molecular weight do we find anything like a one to one relationship between  $1/\Phi$  and the number of aromatic residues.

A number of possible reasons may be responsible for low quantum yields in proteins (222). One of the most important factors involved is the quantum yield for the bond broken in the protein in the primary process. It is reasonably certain that peptide bonds, —S—S— bonds, and aromatic rings are split or oxidized as the protein absorbs light. The final efficiency of splitting of these bonds



TABLE X  
QUANTUM YIELDS FOR INACTIVATION OF ENZYMES, HORMONES, AND VIRUSES AT 2537 Å.

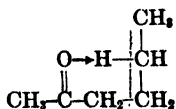
Protein	pH during irradiation	Substrate for assay	Quantum yield	Molecular weight	Quanta per molecule	Aromatic residues per molecule	Ref. No.
Bacteriophage-T <sub>2</sub>	—	—	0.00015	—	—	—	367a
Carboxypeptidase	8.2	Chloracetyltyrosine	0.0014	31,600	715	—	205
Chymotrypsin	4.65	Casein	0.0032	41,000	310	24	120
Hemocyanin	—	—	0.0002— 0.0005*	6.7 × 10 <sup>6</sup>	—	—	—
Insulin	4.0	Mice	0.015	36,000	67	27	187
Pepsin	2.01	Hemoglobin	0.0024	35,500	417	33	226
Ribonuclease	4.1	Yeast nucleic acid	0.026	15,000	38	12	136
Soybean trypsin inhibitor	3.15	Trypsin	0.0088	24,000	114	8	190
Tobacco mosaic virus	7.0	Tobacco	0.000043	42 × 10 <sup>6</sup>	30,000	15,000	225
Trypsin	4.5	Casein (titration), 4.5 Benzoylarginin- amide	0.019	36,500	—	—	351
	4.5	Casein (spectro- photometric)	0.017	—	59	20	349,351
Urease	6.5	Urea	0.01 0.00093	— 483,000	— 1,080	—	189 198

\* At 2553 Å. (Claessen, N., private communication, from the laboratory of T. Svedberg, 1948).

in large molecules may depend upon at least two opposing mechanisms: the Franck-Rabinowitch principle and the principle of pre-dissociation. When dissociation is brought about by certain wavelengths in the gas phase, the same wavelengths will doubtless produce dissociation in the liquid phase or in a monolayer (253). In the monolayer, however, and more so in the liquid phase, the solvent molecules present can collide with the photoactivated molecules, usurp the energy of excitation, and degrade it into heat. This will result in lower quantum yields. In solution with large molecules, where the absorbing group may be near the "center of the molecule" the Franck-Rabinowitch mechanism becomes effective when neither of the radical products is small. The effect of the solvent molecules is in preventing complete separation of the dissociation products (292). This will also result in a lowered quantum yield unless the fragments can react directly with the solvent (253). Even when the fragments of the molecules become fairly well separated diffusion will be slow and the opportunity for recombination will be large unless the fragments fly apart with high kinetic energy. Thus, an increase in frequency may result in a higher quantum efficiency in liquid phase reactions while no such effect is noticed in the gas phase at ordinary pressures. This may partially account for the increase in  $\phi$  with increasing frequency in Landen's results with urease. (It is conceivable that part of these same results may be due to breakage of other bonds in the molecule at shorter wavelengths, these other bonds having an inherently greater quantum yield.) If atomic hydrogen is liberated as a primary product (which seems to be the case for carboxylic groups and amino groups) the liquid state might not be very effective in decreasing the quantum yield. On the other hand, when the radical products are large the effect should be very evident (68). It is quite possible that with protein molecules held together at many points there may be an internal Franck-Rabinowitch effect operating, *i.e.*, two free radical fragments may be held in place by the protein framework until recombination can take place. Ribonuclease, of low molecular weight, has a high quantum yield and its absorption curve changes markedly with photochemical inactivation. Tobacco mosaic virus has a very low quantum yield for inactivation and no changes in physical or chemical properties have been found to accompany loss in infectivity.

In large molecules internal deactivation is probable. An excited potential energy-distance curve of two radicals may cross with a high vibrational level of a ground curve. A transfer at this point results in a molecule in a highly vibrating form which rapidly comes into equilibrium with rotational and translational energies by collisions with other molecules (59). In cases where predissociation may be brought about by foreign fields or by collisions, the primary quantum yield will be increased by changing from the gas to the liquid phase since effectively every absorbing molecule is now in a foreign field. "The distinction between direct dissociation and predissociation all but vanishes under these conditions" (253). Large protein molecules provide a strong intramolecular field which should tend to increase quantum yields over values for bond breakage in small molecules in the absence of foreign fields or molecules. In sufficiently complicated molecules the minimum energy which must be absorbed to break a bond may be less than the bond strength, by virtue of a utilization of the energy in the other degrees of freedom; however, cases (*e.g.*, sulfur dioxide) are known in which the energy which must be absorbed to break a bond may be considerably greater than the minimum required according to thermal data (292). The latter phenomenon seems to be the case with proteins since all but C—H bonds could be broken by the energy per einstein associated with the threshold energy for protein denaturation, 3300–3000 Å.

Another factor which may play a part in the photochemistry of large molecules is that of "internal photosensitizations" in the sense that the bond broken is located in a different part of the molecule from that primarily concerned in the absorption of the quantum (99,206,252,364). A relatively simple example is the conversion of methyl butyl ketone into acetone and propylene at 2200–3000 Å., a region in which the CO group is the chromophore. A mechanism postulated is that of cleavage in a single step as indicated:



Another example involves the photohydrolysis of the  $\omega$ -phenylacyl derivatives of alanine discussed above. The large, folded structure

of the intact, globular protein molecule should provide many possibilities for "internal photosensitization." Many of the aromatic, highly absorbing residues are probably near weak ( $-S-S-$ , or  $-CONH-$ , etc.) bonds whereat a transfer of absorbed energy can occur with cleavage of the weak bond.

Anslow believes that the strong absorption which starts in proteins near 2400 Å. and which is shifted about 3000 wave numbers in strong alkaline solutions is in some way associated with a protein fabric. She believes that if the protein fabric absorbs in this shorter wavelength region the quantum yields for denaturation should increase markedly when proteins are irradiated with UV light of wavelengths 2200–2400 Å. (13). Experimentally, urease exhibits a tremendous change in quantum yield at wavelengths shorter than 2200 Å. but whether or not Anslow's discussion is meaningful awaits further study.

There are doubtless other reasons for low quantum yields in proteins. A screening of functional groups or vital bonds by chromophores other than those associated with activity may exist. It is possible that the quantum yields observed are not the reflection of a primary, bond-splitting process but involve (a) the breakage of a bond not broken in ordinary heat denaturation and (b) conversion of the protein into a "new" protein which is more readily denatured by heat than the original protein (19). One cannot calculate the minimum energy, *i.e.*, the minimum wavelength, of the light necessary to break each bond which is broken by light; of the bonds broken in light denaturation, some are broken thermally, since complete denaturation does not take place at 0° by irradiation alone, at least in egg albumen (87), but only after the irradiated solution has been warmed. The bonds broken by light are probably broken one at a time and these bonds may not be the same as those broken by thermal denaturation in the dark.

## VI. Conclusions

Almost every tool or measurement brought to bear on proteins irradiated by UV light has resulted in noted changes in physical and chemical properties. At present virtually nothing is known about the mechanism of the primary process in inactivation or denaturation by UV light. It is now reasonably certain that  $-CONH-$  and

—S—S— bonds may be broken during denaturation and after denaturation on further irradiation (45,70,206,234,235,237,290). It also seems that the conversion of an enzyme to the inactive state is an all-or-none process accompanied by drastic irreversible changes (88,227,249,310,315). Denaturation requires wavelengths shorter than about 3100 Å. in the absence of a photosensitizer. In general, quantum yields increase with decreasing wavelengths. The quantum yield is also strongly pH dependent but independent of the presence of oxygen. Photochemical processes subsequent to the primary step of inactivation or denaturation are modified by the presence of oxygen.

Further work in this field might well be directed toward a study of the photochemistry of model substances and the organic chemistry of the primary process of inactivation.

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# THE NATURE OF VIRUSES

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## I. Introduction

Viruses are disease-producing entities which resemble pathogenic bacteria to a considerable extent but which are usually differentiated from such bacteria in three important respects. First, most viruses are much smaller than most bacteria. Second, as far as is known, viruses cannot be propagated on simple synthetic media of the type used for the propagation of many bacteria. Third, the diseases produced by viruses, especially those of plants, are usually considerably different from those produced by other agents.

Many virus diseases of man, lower animals, plants, and even bacteria are now known, but the first virus diseases were recognized just before the beginning of the present century. For several decades thereafter relatively little information was available concerning the nature of their etiologic agents. These agents were recognized principally as submicroscopic filter passers capable of causing disease. They were not amenable to study either by the microscope or

by chemical means, for their size range is intermediate between that capable of resolution with the microscope and that of the most complex compounds susceptible to study by conventional chemical techniques. In recent years, however, physical tools have been developed for the investigation of particles within this range of size. With the electron microscope it is now possible to obtain images of virus particles. Through the methods of ultracentrifugation, diffusion, stream double refraction, and viscometry, it is now possible to learn much about the size, shape, and state of hydration of virus particles. It is now possible to obtain some viruses in relatively pure form in sufficient quantity to be analyzed by microchemical techniques. As a result of these advancements, much information has become available during the past decade concerning the physical and chemical nature of viruses. At least some viruses seem to be only a little more complex than some of the larger protein molecules, and the concept that viruses are protein molecules has been extremely useful to the development of knowledge concerning these agents. However convenient this concept may be, viruses are differentiated from most proteins by virtue of their ability to multiply in a manner simulating that involved in reproduction of simple forms of life. Because of this obvious kinship with recognized living agents, no characterization of a virus is complete unless it includes an elucidation of its biological as well as its physical and chemical characteristics. It is thus evident that the nature of viruses can be revealed only through the use of varied techniques: physical, chemical, and biological.

In the preparation of this review, an attempt has been made to discuss some of the important contributions made by biologists, biophysicists, and biochemists to an understanding of viruses. So many studies have been carried out in recent years that a comprehensive review would occupy many times the space allotted to this chapter unless it were reduced to little more than a list of references. It was, therefore, necessary to limit the discussion to certain aspects of the question of the nature of viruses. Many important and interesting studies have not been included. More is written concerning the nature of viruses causing diseases of plants and of bacteria than concerning the nature of viruses which produce diseases of man and animals. This is due, at least in part, to the greater ease with which the plant and bacterial viruses can be studied. In

the first place, the biological characteristics of plant and bacterial viruses can be investigated much more economically than those of the animal viruses. In the second place, the plant and bacterial viruses can be obtained in relatively pure form much more easily than the animal viruses and, for this reason, can be subjected much more readily to study by physical and chemical techniques.

## II. Infection of Host Cell

### A. PLANT VIRUSES

It was pointed out by Anderson (1) in 1946 that the life cycle of viruses has three parts. The first of these is infection, attack, or specific adsorption on a host cell, including penetration into the host cell. The second is development in the cell, including reproduction or multiplication, with attendant specific host cell responses and effects, followed by liberation. The third is a free or dormant state. Most of the direct evidence regarding the nature of viruses derives from studies on the virus in the dormant state. This evidence may or may not provide a true picture of the virus as it exists in other stages in the host. Evidence on the nature of viruses in the first and second stages is necessarily indirect because of their small size.

Plant viruses can be divided roughly into three groups as regards infection: first, those that cannot be transmitted from host to host by mechanical means but which can be transmitted by insects and by one or another form of grafting; second, those that can be transmitted mechanically through wounds made in the presence of the virus by the puncture of insects or in some other manner; third, bacterial viruses which seem able to infect their hosts in the absence of wounds.

There is considerable controversy at the moment regarding the role of insects in transmission of plant viruses. It is generally agreed, however, that the insect-transmitted virus may be either persistent or nonpersistent in its vector. Persistent viruses undergo an incubation or waiting period in the insect after it feeds on a diseased plant before being capable of transmission to a new host. The insect vector is then able to transmit for days, weeks, or even months. Nonpersistent viruses can be transmitted immediately after feeding on a diseased plant. The insect vector soon loses the ability to transmit nonpersistent viruses unless again fed on diseased plants.



Aster yellows virus has been shown to be transmitted by the leafhopper *Macrostelus divinus* Uhler after an incubation period of from ten to seventeen days (74) and is retained in the vector for life. Kunkel (76,77) has shown that viruliferous leafhoppers lose the ability to transmit when exposed to a temperature of 90°F. for one day. When held at this temperature for a period of from one to eleven days, insects lost the ability to transmit but they regained the ability, without again feeding on a diseased plant, when the temperature was lowered to about 75°F. The speed with which they regained ability to transmit was inversely proportional to the length of time held at the high temperature. When held for twelve days or longer at 90°F., the insect completely lost the ability to transmit. Kunkel interpreted these results as due to virus inactivation at the high temperature, followed by multiplication of virus at the lower temperature. Black (23) found that aster yellows virus could be transmitted from leafhopper to leafhopper by mechanical inoculation and that insects so inoculated were able to transmit the virus to asters after a suitable incubation period. Mechanical transmission from insect to insect was used as a means of testing for presence of virus in insects that had fed on diseased plants for two days and were then held on nonsusceptible rye plants. Black was able to recover the virus four or more days after the insects had fed on diseased plants, but failed to pick it up during the first three days after feeding. Generally, insects tested twelve or more days after feeding on diseased plants had a higher titer than those tested after a shorter time. Black interpreted these results as evidence for multiplication of aster yellows virus in its insect vector.

Bawden (9) offered a different interpretation of Black's data. He pointed out that, according to the data, the virus was more readily transmitted from insect to insect when the juices were diluted  $10^{-3}$  than when diluted  $10^{-2}$  or  $10^{-1}$ . This, he believed might be due to the presence of the inhibitor which Black (22) had previously found in the aster leafhopper. Activity of this inhibitor was reduced by dilution, thus freeing the virus and accounting for more infections at  $10^{-3}$ . The increase in titer of virus in the insect, as length of time after feeding on a diseased plant increased, might be due, according to Bawden, to the inhibitor's becoming less and less active during the incubation period.

Fukushi (46,47) has shown the virus of dwarf disease of rice to

be transmitted through eggs of its insect vector, *Nephotettix apicaulis* Motsch. In subsequent papers (48,49) he showed that the virus was retained for 374 days within insects of five generations and, in one case to the seventh generation. Black (24) found that clover club leaf virus was transmitted through eggs to 89% of the living progeny of viruliferous females. In no case, however, did the nymphs transmit the virus until at least three weeks after hatching. Transmission of virus through several generations of insects by passage through eggs could hardly be accomplished in the absence of multiplication within the insect body, for without multiplication the dilution factor would be so great as to render the probability of finding virus present infinitely small. Fukushi's results therefore constitute convincing evidence for multiplication within the vector and Black's result with clover club leaf virus supports the evidence.

In contrast, Freitag (44) and Bennett and Wallace (18) have presented evidence that sugar beet curly top virus does not multiply in its vector *Eutettix tenellus*. This virus has only a short incubation period in its vector, a period of four hours being sufficient for the insect to pick up the virus and transmit it to a healthy plant (18). The vector, however, may retain ability to transmit for many days (167). When fed on a series of healthy test plants in succession or on resistant plants, the vectors gradually lose ability to transmit (18,44). The ability to transmit has been found to be correlated with the length of feeding on diseased plants, indicating that the initial amount of virus acquired by the vector determines its ability to transmit (18,44). These observations have led to the conclusion that the curly top virus probably does not multiply in its vector.

Watson (197) compared transmission of a persistent and a non-persistent virus by the same species of aphid. She pointed out that insect-transmitted viruses cannot be differentiated sharply on the basis of persistence in their vectors, for the incubation or waiting period varies from a few hours, or minutes, to many days, and the length of time the vector is able to transmit likewise varies.

The only possible way at the moment to reconcile these conflicting views regarding the multiplication of so-called persistent viruses in their vectors is to assume that some persistent viruses, such as that of sugar beet curly top, multiply little if any in their vectors, while others, such as that of aster yellows, do multiply to a considerable extent. It would seem of importance to settle this question

definitely since the susceptibility of an insect to a plant virus would provide the only case known in which viruses paratitimize both animals and plants.

The most effective way of inoculating plants with most mechanically transmitted viruses is to rub the virus solution across the leaf surface. Rubbing in the presence of the virus is essential in order to provide contact between the virus and an injured cell. It was long believed that the most common point of entry of the virus was through an injured trichome. However, Boyle and McKinney (25) have shown that other injuries on the leaf surface are more important as centers of infection. Samuel and Bald (160) found that 120 mesh sand used as an abrasive, increased the number of infections with a yellow mottling strain of tobacco mosaic virus. Rawlins and Tompkins (157,158) showed that the use of fine carborundum powder was effective in increasing the numbers of infections with several plant viruses. They believed that the carborundum crystals in many cases pierce the cells in such a manner as to allow virus to enter and multiply.

When any one of a number of plant viruses are rubbed across leaves of appropriate host plants, necrotic local lesions are produced in a few days time (56,156,203). The numbers of such lesions are a function of the virus concentration of the inoculum, and Holmes (56) originally pointed out their usefulness for measurement of virus activity. Lauffer and Price (99) studied the relationship between virus concentration and numbers of lesions in an attempt to elucidate the nature of infection by mechanically transmissible viruses. Youden, Beale, and Guthrie (202) and Bald (5,6) had previously shown that the general equation  $y/N = 1 - e^{-vnx}$  (where  $y$  is the number of lesions produced,  $N$  is the theoretical maximum number or the number of susceptible areas,  $v$  is a small volume,  $n$  is the number of virus particles per milliliter of undiluted inoculum, and  $x$  is the dilution factor) could be used for plotting the dilution curves for several plant viruses. Parker (143) had found it satisfactory for interpreting dilution curves for vaccinia virus. This equation was derived from the Poisson series on the assumption that infection of a plant or an animal could be induced by a single virus particle. Bryan and Beard (26), on the other hand, have postulated that infection with viruses is of an entirely different type, being similar to the response of organisms to different dosages

of drugs. According to their view, a great number of virus particles coming into contact with susceptible host cells are combatted by the defense mechanism of the host, a defense mechanism which varies with the host. When the number of virus particles is sufficiently great the defense mechanism is overcome and the host becomes infected. Lauffer and Price found that the dilution curve for many host-virus combinations could be satisfied by the same dilution equation. In order for the theory of Bryan and Beard to hold for all these combinations, it is necessary for the standard deviation of host susceptibility to be a universal constant—a very unlikely possibility. Moreover, it has been shown that the number of lesions resulting from mixed infections after inoculation with various dilutions of a mixture of two viruses was more consistent with the theoretical number predicted from the Poisson curve than with the number predicted from the curve of Bryan and Beard. The study of Lauffer and Price therefore led to the conclusion that infection can be caused by a single infectious unit.

In a recent review paper, Beard, discussing this work (15), made the statement that: "obviously, one infectious unit, by definition, will initiate an infection." That, of course, depends upon how one defines an infectious unit. It seems clear that the terms single infectious unit and single virus particle are used synonymously by Lauffer and Price. Studies on purified virus preparations by many investigators have revealed that viruses are made up of particles more or less uniform in size and shape. There is no proof that all these particles are active, since we are far from obtaining a one to one ratio between the number of particles and the number of infections. The question is whether one of these particles, if active and favorably situated with respect to the host, can induce infection or whether more than one must be present. According to Lauffer and Price, only one such particle is required. According to Bryan and Beard a variable number of particles are required, depending upon the susceptibility of the host.

#### B. BACTERIAL VIRUSES

Infection of bacteria with bacteriophage is somewhat more complicated than infection of higher plants with viruses. According to Delbrück (33) lysis of a sensitive strain of *Escherichia coli* by phage may be either from within or from without. Lysis from within

occurs when a bacterium is infected by one or a few virus particles which then multiply within the bacterial cell. When the virus has reached a threshold value it is released by sudden destruction of the protoplasmic membrane. The cell contents are exuded without deformation of the cell wall. Lysis from without occurs instantaneously by adsorption of phage onto the cell wall. The bacterial cell swells and becomes deformed into a spherical body. This sort of lysis occurs only when the number of virus particles greatly exceeds the number of bacterial cells exposed. There is no multiplication of phage in this case. Delbrück is of the opinion that lysis of these or other bacteria by viruses is of one of the types described above.

There is abundant evidence that only a single bacterial virus particle is required to initiate infection. For example, Delbrück and Luria (37) compared theoretical and experimental values for numbers of bacteria surviving when a given concentration of bacteria was exposed to varying amounts of virus. The survival values were calculated on the assumption that only a single particle is required to infect. Excellent agreement between experimental and calculated values was obtained.

As pointed out earlier, viruses are seemingly able to infect bacteria in the absence of wounds. There is some sort of an attraction between the virus particles and the susceptible bacterial cell. Adsorption of phage to bacteria, which precedes actual infection, has been the subject of a number of studies. The rate of adsorption is a function of both the bacterial concentration\* and the phage concentration. It is also affected by the salt concentration of the mixture (73,166), and by the physiological state of the bacterium (32). Although the rate of adsorption may vary for different experimental conditions, the total amount of phage that can be adsorbed by a bacterium is more or less fixed. One of the interesting things about this threshold of adsorption is that the capacity to adsorb is almost exactly equivalent to the maximum yield of virus from within the cell after multiplication and lysis have taken place (33). This observation has led Delbrück (33) to suggest the possibility that the bacterial constituent which adsorbs the phage may be part of a precursor which, under favorable conditions, is transformed into free phage and which accounts for multiplication of the phage.

It has been found that certain "cofactors," such as L-tryptophan,

have the power of promoting adsorption of certain bacteriophages on their hosts. Anderson (2) has shown that it is the virus, not the bacterial cell, that is activated by the cofactor. He also found that the effect of L-tryptophan on the adsorption of T4 virus by *Escherichia coli* is reversible. At 15°C. *E. coli* is resistant to some viruses that are active on it at 37°. Anderson (4) obtained results which suggest that this is due to an insufficient supply of cofactor at 15°, an insufficiency which could be overcome by addition of certain amino acids to the medium. The degree of requirement for cofactor is inherited by the virus (3).

### III. Growth and Reproduction in Host Cells

Experimental evidence regarding growth and reproduction of viruses within their hosts is necessarily meager because of the difficulty of distinguishing between virus activity and host cell activity. That viruses reproduce is certain, but the role played by the host in reproduction is unknown. If a solution of Southern bean mosaic virus is rubbed over leaves of a Golden Cluster bean plant grown at a fairly high temperature, necrotic local lesions develop in a minimum period of 28 hours. At this stage, each lesion has a volume of about one cubic millimeter. It has been shown that mottled leaves of Bountiful bean plants contain about 0.4 mg. of virus per milliliter of sap (152), and it can be assumed that infected areas of the Golden Cluster bean have an equivalent concentration. Since the spherical particles of Southern bean mosaic virus weigh  $2 \times 10^{-16}$  g. (131), it can be calculated that the local lesion of one cubic milliliter volume contains  $2 \times 10^{12}$  particles. In many cases, such lesions originate from a single virus particle. We can therefore say that the virus has increased from 1 to  $2 \times 10^{12}$  particles in a period of 28 hours. If it is assumed that reproduction has taken place by division of the original particle into two, and each of these into two and so on, the curve may be represented by the simple exponential  $2^n$ . For the case cited  $n = 41$ . This means that a doubling of the number of virus particles every forty minutes would account for the presence of 0.4 mg. of virus in a 28 hour old lesion. This value is in good agreement with that found for many bacteria.

The above calculation by no means proves that viruses reproduce

by successive divisions. Yet the growth curve for reproduction of viruses is sigmoidal in shape as is indicated by data of Holmes (57), among others. Such curves cannot be interpreted exactly, for they represent multiplication plus movement in a multicellular organism. They are actually composites, representing the summation of growth curves in individual cells. For this reason, they have not so far thrown very much light upon the nature of the mechanism of virus reproduction.

Growth curves of bacteriophages in their hosts are somewhat better in this respect for, although they too involve growth in a large number of bacterial cells, they are not complicated by movement of virus from cell to cell. However, they are complicated by other factors which make it difficult to determine actual rates of virus reproduction. As shown by Delbrück (33), when phage is added to a bacterial culture in quantities such that more bacteria than phages are present, there is a latent period during which no increase in free phage can be detected. This period is followed by another during which virus is liberated at a constant rate, and finally by a third period during which no increase in phage can be detected. These observations are interpreted in the following manner: during the latent period, virus multiplies but is not free and cannot be detected; after the latent period, the bacteria burst, liberating free phage. Liberation occurs at a constant rate until all infected bacteria have been lysed. While this sort of data is valuable to the experimenter in helping to study questions of adsorption of virus onto bacteria, it does not give a real insight into the question of virus multiplication.

#### IV. Virus Mutations

It has been known for a long time that viruses exist in nature in the form of strains or variants. Over 400 different strains of tobacco mosaic virus have been recognized (79). In 1933, Jensen (61) offered proof that new strains of tobacco mosaic virus arise by mutation or a similar process during multiplication of other strains in their hosts. Similar evidence for the origin of strains of cucumber mosaic virus was found a year later by Price (150). The proof for mutation of plant viruses is conclusive. McKinney (122) was the first to show that variant strains of tobacco mosaic virus could be

isolated from yellow spots that occur on tobacco plants infected with a green mottling type strain. At this time there was no evidence that the green mottling type strain was pure. Jensen passed ordinary tobacco mosaic virus through a series of ten successive local lesions on the assumption that most of the local lesions were initiated by a single virus particle and, therefore, that the probability of obtaining a mixed infection after ten passages would be practically zero. A pure line of virus isolated in this manner still gave rise to variants. Since it is overwhelmingly probable that the variants were not present to begin with, they must have arisen from the pure line strain. Jensen's results have been amply confirmed for this and other viruses during the succeeding years.

Kunkel (77) has enumerated the various strains of tobacco mosaic, cucumber mosaic, potato latent mosaic, beet curly top, sugar cane mosaic, and a few other viruses that have been found in nature or obtained by mutation in the laboratory. He makes the interesting observation that some viruses have large families of variants while others have small families. This fact indicates that viruses differ in ability to mutate. Even strains of the same virus differ in mutability. Kunkel (78) found that the rate of production of yellow spots on plants inoculated with four different strains of tobacco mosaic virus varied with the strain, indicating a difference in ability of the four strains to mutate in a given direction.

The most used method of obtaining variants is to isolate them from yellow spots occurring on infected host plants. Passage of virus through resistant hosts is another method for obtaining variants (63,80-84,196). Host passage is used frequently by virologists to obtain virus strains to serve as vaccines. Allowing virus to reproduce under abnormal temperature conditions is a third method for obtaining variants (62,58). It is a controversial question at the moment whether such methods cause virus mutations or merely serve to separate virus strains already present.

Kunkel (79) has summarized the ways in which variant strains differ from the parent strain. A few of these are: symptomatology, severity of disease produced, amounts and kinds of amino acids, temperature range for multiplication, invasiveness, rate of movement in hosts, and specific insect vectors. Studies of Holmes (59), Norval (137), and Kunkel (75) have shown that certain factors



possessed by tobacco mosaic virus vary independently. For example, Kunkel's studies showed that a factor for green mottling in tobacco and tomato varied independently from a factor for localization in *Nicotiana sylvestris*. If such factors vary independently, and there are at least six that do, they must occupy different sites in the virus particle. Holmes has suggested that such factors represent unit differences in the genetic structure of higher plants and animals. At one time or another viruses have been likened to genes (38,133,137). It has been said that viruses may be genes that have gone wild, acquiring in the process the ability to exist independently in the cell and to move from cell to cell. Kunkel (79) has pointed out that a gene represents a single factor in reproduction, whereas viruses carry a series of factors capable of independent variation. He suggests that viruses behave in this respect more like chromosomes than genes.

It seems quite likely that a single mutation in a virus involves a single unit factor change. Some of the wild strains existing in nature differ more from one another in host range, symptomatology, and other respects than laboratory strains. They probably have been derived by a series of mutations involving not one but many unit factors. Laboratory strains, on the other hand, result from single step, or at most few step, mutations.

Knight (70) has recently shown significant differences in amino acid composition among strains of tobacco mosaic virus. While Knight's work will be discussed in greater detail in a subsequent section, some of its implications are pertinent to the present discussion. He has suggested that each mutation in a virus involves a change in amino acid composition. It is too early to say whether or not this suggestion is correct. If it is, every strain should differ from every other strain in amino acid composition. Knight could not find significant differences among some of the strains tested. This does not mean that such differences do not exist, for methods available do not allow for detection of very small differences. On the other hand, strains could differ in size without differences in relative amounts of amino acids, or they could differ in arrangement of their constituent amino acids. Obviously, if tobacco mosaic virus is a nucleoprotein, strains of the virus must differ from one another in kind, total amount, relative amount, or arrangement of amino acids, or in the nucleic acid fraction of the particle. Study of the

amino acid composition of viruses is a field that merits a great deal more attention.

Bacterial viruses have been found to differ in host specificity, type of plaque produced in susceptible hosts, antigenic specificity (54), and in cofactor requirements (2). According to Luria (116), statistical evidence shows that new virus particles arise by mutation in the course of multiplication of normal virus in the cell. In some respects, more is known about mutation and inheritance in bacterial viruses than in viruses that attack higher plants. Hershey (54) points out that a mutation in T2H bacteriophage affecting its host range occurs once in  $4 \times 10^8$  duplications. A factor for lysis inhibition occurs with a frequency of about  $10^{-3}$  in T2 bacteriophage, whereas the reverse mutation has a frequency of  $10^{-8}$ . Factors affecting host range, lysis inhibition, and antigenic specificity vary independently and therefore occupy independent genetic sites.

Hershey (54) studied two mutable characters in T2 bacteriophage. One of these was concerned with host range, the other with type of plaque produced. His experiments provide evidence that each of these characters is determined by several sites, or loci, capable of independent mutation. At each locus there seems to be a single structure subject to reversible alteration between only two contrasting characters. Hershey was unable to find evidence for multiple characters occupying the same genetic locus, for mutation by loss of a factor, or for mutation involving two or more factors simultaneously. Anderson (3) has shown that requirements for adsorption cofactors are inherited by T4 bacteriophage.

It has been found that, when a bacterium is inoculated with two different viruses, only one will generally be recovered (34,37,117). This has become known as the interference phenomenon or exclusion effect. Delbrück and Luria (37) point out that two closely related viruses will interfere in both directions (mutual exclusion) while two unrelated viruses will interfere either in only one direction or in neither. It was shown by Luria and Delbrück (117) that a single particle of virus inactivated by ultraviolet irradiation will suppress the growth of another virus in a bacterium. They suggest that interference is the result of competition for a key enzyme present to a limited extent in the bacterial cell.

Delbrück and Bailey (36) carried out experiments with wild and mutant types of T2 and T4 bacteriophages of *Escherichia coli*.

When appropriate mixtures were used, not only both elements of the mixture but also a new type of virus could be recovered. They suggested two possible explanations for these results: (1) the presence of one type of virus had induced mutations in the other, and (2) an actual transfer or exchange of genetic material had taken place.

Studies on inheritance in viruses have shown clearly that these entities are more highly organized than genes that control the heredity of higher plants and animals. They must themselves contain a number of genes or genelike structures. Further research along these lines may be expected to yield information about the nature of these structures. For example, most of the studies have so far dealt with viruses of sufficient volume to contain thirty or more genes like those of *Drosophila*. If some of the smaller viruses could be shown to contain a number of factors independently inherited, these factors would have to be different from genes of higher organisms, or the genes would have to be smaller than is now supposed. In any event it is becoming increasingly clear that viruses are not like genes themselves but may resemble groups of genes such as may be found in a fragment of a chromosome.

## V. Purification and Crystallization of Viruses

More progress has been made during the past decade than in any other comparable period of time in the elucidation of the nature of viruses. To a very considerable extent, this can be attributed to the development of methods of purification. Great impetus for the development of such techniques was afforded by the crystallization and characterization of tobacco mosaic virus by Stanley (182). The demonstration that tobacco mosaic virus could be crystallized by methods previously used in the study of proteins and that it was a nucleoprotein (10,113,185) resulted in the application of these techniques to the purification of many other viruses. Included are precipitation by electrolytes, precipitation by water-miscible organic solvents, isoelectric precipitation, and adsorption onto and elution from active surfaces. All these procedures have been used in the purification of viruses. The demonstration that tobacco mosaic virus protein had an unusually high molecular weight suggested the pos-

sibility that this virus could be purified by means of high speed centrifugation. Accordingly, the technique of centrifugation was developed for this purpose (200,201). This method had been used previously in the purification of several viruses (8,110,119,121,144). Today, a favorite method for purification of all sorts of viruses is that of differential centrifugation.\* A suspension containing minced plant or animal tissue or an extract of diseased tissue is first centrifuged at low speed to remove large particles and then at high speed to remove the virus. The virus is further purified by alternate high and low speed centrifugations. This method is particularly useful because it is relatively less severe than the chemical methods and can be used to purify even fairly unstable viruses. Advantage has also been taken of the migration of viruses in an electric field. The Tiselius electrophoresis apparatus has been used in the purification of Southern bean mosaic virus (101).

The technique of high speed centrifugation as applied to the purification of viruses has one very serious limitation. The angle centrifuge is not a sensitive device for fractionation of particles. This can be illustrated by experience with influenza virus (108). By the application of two centrifugation cycles involving centrifugation at 24,000 r.p.m. for thirty minutes and centrifugation at 5000 r.p.m. for about the same period of time, a preparation was isolated from the allantoic fluid of chicken embryos diseased with PR8 influenza A virus. This material consisted largely of particles with a sedimentation rate of about 700 Svedberg units. However, a small amount of material with sedimentation rate about a third of that was also shown to be present by a viscosity technique. This preparation was fractionated by a procedure which involved ten short runs in the high speed centrifuge and various recombinations of fractions. Even after this drastic fractionation procedure, a small amount of the more slowly sedimentating material remained as a contaminant. This illustrates the difficulty of separating materials by the technique of fractional centrifugation. The success of the

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\*The use of the term "differential centrifugation" for the process of alternate high and low speed centrifugation has been criticized by Beard (15) on the basis that it implies that the method is highly sensitive as a fractionating device. It is difficult to understand why such a restricted definition of the term "differential centrifugation" should be accepted, but it is agreed that the method as commonly employed is not a sensitive fractionating procedure.

centrifugation technique depends upon the availability of a component within diseased tissue which has a sedimentation rate vastly different from other components of the same tissues. In the case of studies with plant viruses, the centrifugation technique has been successful largely because the normal proteins of the plant cell have much lower sedimentation rates than viruses and the cellular debris generally has a much higher sedimentation rate.

It has been suggested that the ability to obtain by means of centrifugation preparations of virus which are relatively homogeneous in the ultracentrifuge is attributable to the technical skill of the investigator and not to the state of the virus in extracts of diseased tissue (146). This point of view would be reasonable if an angle centrifuge and an analytical ultracentrifuge were equally sensitive to inhomogeneity. Beard (15) and Markham (123) have recognized the importance of this distinction, but it has been overlooked by many. A preparation of tobacco mosaic virus has been described which consisted of particles with a single mean sedimentation rate and with a distribution of sedimentation rates described by a standard deviation equal to 4% of the mean, as determined in the analytical ultracentrifuge (89). It would be virtually impossible to obtain such a degree of homogeneity by any complex centrifugation technique in an angle centrifuge, if one started from a supply with an infinite distribution of particle sizes. Such a distribution could be obtained only because in the supply a constituent was present with essentially that degree of homogeneity. All larger particles must have been very much larger and all smaller particles must have been very much smaller. The experiments of Smadel, Pickels, Shedlovsky, and Rivers (180) on the fractionation of collodion particles by means of ultracentrifugation bear out this contention. They were able to obtain a fraction with a mean sedimentation rate approximately the same as that of vaccinia virus, which could be isolated by the same technique. However, their sedimentation diagrams show that the distribution of sizes in the fractionated collodion was very much greater than that in the virus preparation.

Many viruses have been obtained in purified form. Five have been obtained in crystalline form. These include tobacco mosaic virus and several of its strains, tobacco necrosis virus (13,147,154), tomato bushy stunt virus (11,187), Southern bean mosaic virus

(152), and turnip yellow mosaic virus (124). Purified preparations of other viruses have been reported. These include vaccinia virus (144), influenza virus (108,193), mouse encephalomyelitis virus (50), various strains of poliomyelitis virus (52,115), Newcastle virus (7), equine encephalomyelitis virus (192), rabbit papilloma virus (7), and certain types of bacteriophage (60,118).

## VI. Characterization of Virus Preparations

### A. PHYSICAL CONSTANTS

Virus preparations can be characterized by certain physical constants. Frequently, these are useful for the purpose of determining something about the nature of the virus particle, but in any case, they serve as a means of identification. The ultrafiltration end point, the sedimentation constant, the diffusion constant, the intrinsic viscosity, the specific refractive increment, the isoelectric point, and the electron microscope image size are the most frequently determined constants. Table I contains a list of such constants for eleven purified virus preparations.

### B. IDENTIFICATION OF VIRULENT PRINCIPLE WITH CHARACTERISTIC PARTICLE

For obvious reasons, the most important single question concerning a preparation obtained from virus diseased material is whether the predominating particles found in that preparation really are the bearers of virus activity. Unless they are, detailed study of the preparation is largely an exercise in biochemical and biophysical technique. It is not an easy matter to establish the identity of a virulent agent with the physical particles in any preparation, but it is possible to bring considerable evidence to bear upon the question.

Bacteriologists are confronted with the same problem in establishing the relationship between a bacterium and a disease. Koch postulated that an organism can be considered as an etiologic agent if it fulfills his four well-known criteria: (a) the organism must always be associated with the disease; (b) it must be isolated in pure culture on artificial media; (c) the disease must be reproduced when a suitable host is inoculated with the pure culture; and (d) the organism must be recovered from such a host. Evidence anal-

TABLE I\*

Virus	Sedimentation constant <sup>b</sup>	Diffusion constant <sup>c</sup>	Specific refraction increment <sup>d</sup>	Apparent partial specific volume (V) <sup>e</sup>	Intrinsic viscosity <sup>f</sup>	Filtration endpoint m $\mu$	Electron microscope size, m $\mu$	Isosmotic point, pH
Tobacco mosaic .....	185 (89)	5.3 (89)	0.0017 (85)	0.73 (89)	39.0 (89)	34-45 (194)	15 X 280 (142)	3.49 (41)
Tobacco necrosis .....	112 (154) 49 (138)	—	0.0016 (138)	—	—	40 (181)	27.5 (126)	—
Squash crystal mosaic..	—	—	—	—	—	—	30 (189)	—
Tomato bushy stunt...	132 (106)	11.5 (134)	0.0017 (120)	0.74 (120)	—	40 (181)	25.5 (153)	4.11 (120)
Southern bean mosaic..	115 (131)	13.4 (131)	—	0.70 (131)	6.3 (131)	—	25.0 (153)	5.50 (130)
Turnip yellow mosaic..	—	—	—	0.69 (29)	—	—	19.5 (29)	—
Vaccine .....	4900 (145)	—	—	—	—	250 (39)	210 X 260 (53)	—
PR8 Influenza A .....	722 (108) 742 (175)	—	—	0.79 (108) 0.82 (175)	14.3 (108)	163 (45)	115 (108) 101 (175)	5.3 (129)
Lee Influenza B .....	810 (188) 840 (175)	—	—	—	11.0 (128)	—	—	5.4 (128)
Rabbit papilloma .....	290 (135)	5.85 (135)	—	0.75 (135)	8.2 (135)	—	44 (173)	5.0 (169)
T2 bacteriophage .....	700 (168) 1000 (163)	—	—	0.66 (191)	—	—	—	4.2 (168)

\* Reference numbers are given in parentheses.

<sup>b</sup> S<sub>20</sub><sup>w</sup> X 10<sup>13</sup>, (cm./sec.)/(cm./sec.<sup>2</sup>).<sup>c</sup> D<sub>20</sub><sup>w</sup> X 10<sup>-8</sup> cm., cm.<sup>2</sup>/sec.<sup>d</sup> Cm.<sup>2</sup>/g.<sup>e</sup> Cm.<sup>2</sup>/cm.<sup>2</sup>

ogous to the first, third, and fourth criteria of Koch is available for all the purified virus preparations subjected to any considerable amount of study. The second criterion, however, cannot be applied literally, because viruses cannot be cultivated on artificial media. Nevertheless, various other tests of purity can be applied. At one time it was considered sufficient evidence for the identity of a particle with a virulent agent when the first, third, and fourth criteria of Koch were satisfied literally and when a reasonable degree of evidence was presented for the purity, from a chemical point of view, of the virus preparation. Various chemical and physical criteria of purity have been applied to many virus preparations. Most conspicuous of these are constancy of chemical composition, homogeneity with respect to sedimentation rate and electrophoretic mobility, and uniformity with respect to electron microscope images. However, purity is only relative. All separations by either chemical or physical procedures are, of necessity, partial, and absolute purity from the chemical and physical standpoint is not a realizable goal. Since virus activity measurements are almost always relative, the possibility usually exists, until excluded by other means, that the infectious principle is contained within the small amount of impurity tolerated and is not a property of the predominating material in a relatively pure preparation. Thus, through the natural evolution of the discipline of virology, it is gradually becoming evident that chemical purity can no longer be regarded as a satisfactory criterion of identity of virulent agent and characteristic particle. This difficulty does not apply to Koch's second criterion in its original form, because cultivation on a synthetic medium is a test of the biological purity of a material, a severe one at that, and not a test of the chemical purity.

A more effective way of establishing the identity of a virulent agent with a type of particle is to compare the chemical and physical properties of the virulent agent and of the particles. For example, if it can be shown that the characteristic particles of a virus combine chemically with formaldehyde and also that formaldehyde alters the biological activity of the virus, one has some evidence of the identity of the virus and the particles. It is easy, however, to conceive of chemical reactions with particles which do not alter virus activity, even though virus activity is associated with those particles; it is also easy to conceive of treatments which destroy



virus activity without producing measurable chemical change on the particles bearing the activity. For this reason, this chemical approach is satisfying only when there is abundant evidence of parallelism between the chemical properties of the virulent agent and of the purified preparation of particles.

A more satisfactory chemical approach is one which employs chemical fractionation. For example, the solubility of any material of a protein nature depends upon, or can be altered by changing, the pH of the medium, the kind and quantity of salt in the medium, and the temperature, or by adding various water miscible organic solvents. If, within the range of stability of the virus, the infectious principle possesses the same relative solubility as the suspension of particles, evidence is available for the identity of the characteristic particle and virulent agent. Chemical evidence of both sorts is available in the early work of Stanley (183,186) for the identity of the infectious principle of tobacco mosaic virus and the tobacco mosaic virus nucleoprotein. The principal limitation of chemical techniques of the second sort lies in the relatively low precision with which the experiments are usually carried out.

A potentially very precise approach to the establishment of the identity of a virulent agent and a suspension of particles is the determination of the identity of the physical constants of the virulent agent and those of the suspension of particles. Most of the physical constants useful for such a comparison are obtained by studying the movement of the material under various circumstances. To get the constant for the infectious principle, one must follow that movement solely by infectivity measurements. To get the constant for the characteristic particle, one must follow the migration by purely physical or chemical means. This method has the considerable advantage of being applicable even to impure virus preparations.

If a particle under study is the bearer of virus activity, that particle should sediment in a centrifuge at exactly the same rate as the infectious principle. It should migrate in the electrophoresis apparatus with exactly the same rate as the infectious principle. It should diffuse at the same rate as the infectious principle, and it should have the same filtration end point as the infectious principle. In the case of sedimentation, electrophoresis, and diffusion, it is possible to measure the migration of particles by purely physical

means. The rate of migration of the infectious principle can in all cases be measured by combining physical experiments and infectivity measurements.

The sedimentation constant of purified particles can be measured by the usual optical means with the ultracentrifuge. The sedimentation rate of the infectious principle can be measured relatively crudely through sedimentation studies involving the angle centrifuge and with considerable precision by the technique of sedimentation in a capillary tube or by use of the separation cell of Tiselius, Pedersen, and Svedberg (195). The latter device has been used to establish the identity within a small error of the sedimentation rate of the characteristic tobacco mosaic virus particle and the infectious principle (87), and also of the characteristic influenza virus particle with the infectivity and the red blood cell agglutination activity of the virus (98). The Tiselius electrophoresis apparatus can be used to measure the electrophoretic migration rate of particles by optical means (111,112). Also, samples can be withdrawn for analysis, thus permitting the determination of mobility of the infectious principle. By this means, the mobilities of the red blood cell agglutination activity of influenza virus and of the influenza virus particle were shown to be indistinguishable (129). The mobilities of the characteristic particle and of the infectious principle of Southern bean mosaic virus were also shown to be indistinguishable (101). The diffusion rate, or diffusion constant, of a virus material can be determined by optical means and the diffusion constant of the virulent agent can be determined by the method of Northrop and Anson (136) or by the method of Polson (148). If the virus is really associated with the characteristic particle, the two diffusion constants should be identical. In this manner, Polson (149) determined the diffusion constants of the infectious principles of the T3 and T4 bacteriophages of *Escherichia coli*. Values for  $D_{20}^w$  of 1.19 and 0.08, respectively, were obtained. It is possible to test the ultrafiltrate of a virus suspension both for virus activity and for physical particles. If the experiment is carried out in such a way that the investigator is not misled by the enormous difference in sensitivity between virus activity measurements and chemical techniques, this process can provide additional evidence of the identity of a virulent agent and a particulate entity. Various combinations of the above methods can be used. For example, the diameter of the in-



Fig. 1. Shadowed electron micrograph of tobacco mosaic virus particles (142).

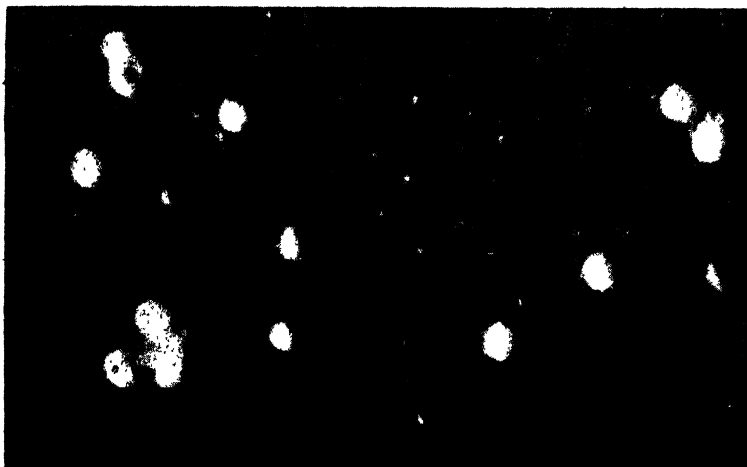


Fig. 2. Shadowed electron micrograph of PR8 influenza A virus particles (198).

fectious agent determined by ultrafiltration can be compared to the diameter of the characteristic particles as determined by electron microscopy or other means. Equivalent diameters of the in-

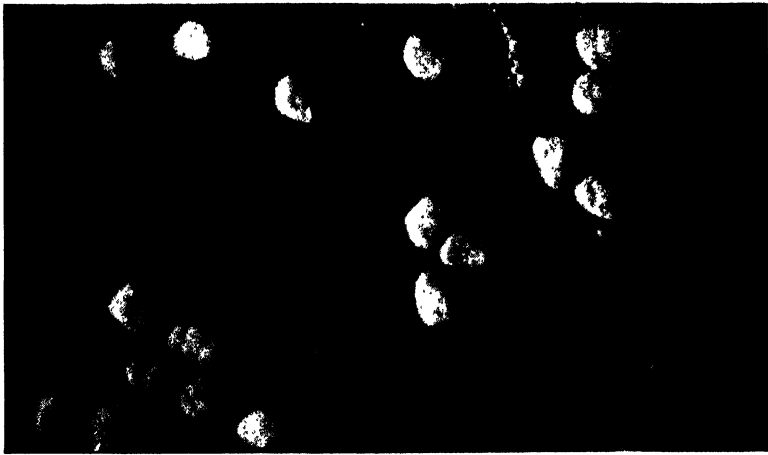


Fig. 3. Shadowed electron micrograph of elementary bodies of vaccinia (173).

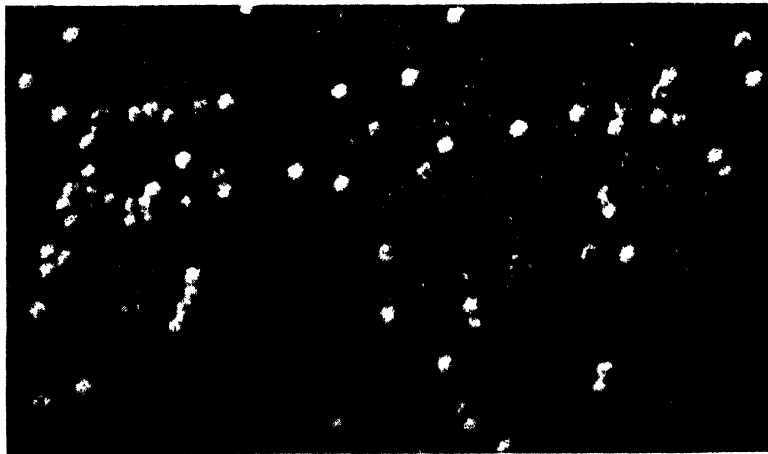


Fig. 4. Shadowed electron micrograph of rabbit papilloma virus (173).

fectious units calculated from Polson's diffusion constants (149) agreed reasonably well with sizes of the electron microscope images of the T3 and T4 bacteriophages (35).

The evidence for the identity of the virulent agent of tobacco mosaic virus and the characteristic rodlike virus particles, such as those shown in Figure 1, includes tests for purity, comparisons of the chemical properties of the virulent agent to those of the particles, and correlations of physical properties of the virulent agent with those of the rodlike particles. This evidence has been reviewed in detail elsewhere (93,183). Similar types of evidence, also reviewed previously (91,93), are available for the identity of PR8 influenza A virus and the hundred millimicron spherical bodies shown in Figure 2. The evidence, previously reviewed (93,178), in favor of the identity of the virulent principle of vaccinia virus and the elementary bodies of vaccinia, shown in Figure 3, is of somewhat the same character but is buttressed by the demonstration that infection can be produced by elementary body suspensions so dilute as to contain very few elementary bodies. This would mean, in this particular case, that, if the infectious principle is attached to an impurity, the impurity must be present to a very considerable extent.

Beard (15) has considered the viruses of equine encephalomyelitis, rabbit papillomatosis, and T2 bacteriophage of *Escherichia coli* to be in the same category with those just discussed. The evidence in the case of rabbit papilloma virus, characteristic particles of which are shown in Figure 4, indicates a reasonable degree of purity (16,17). The pH stability range of the virus coincides relatively well with the pH stability range of the particles in a suspension, and coagulation of the particles by heat destroys infectivity. Furthermore, isoelectric precipitation of the particles results in precipitation of the infectious agent. The sedimentation of the infectious principle in an angle centrifuge proceeds at a rate not incompatible with the sedimentation constant of the purified particles. The evidence in favor of the identity (60,168,191) of the virulent agent and the characteristic particles with respect to the equine encephalomyelitis virus (171) and with respect to the T2 bacteriophage of *E. coli*, shown in Figure 5, consists largely of the same sort. The crystalline plant viruses are all reasonably pure, otherwise crystal formation would not occur. Crystallization, however, is not a sensitive criterion of purity. Sedimentation diagrams obtained with bushy stunt (86), Southern bean mosaic (131), and tobacco necrosis viruses (131) give evidence of homogeneity. In the case of tomato

bushy stunt virus a critical analysis of the sedimentation evidence indicates that this material is of a very high degree of homogeneity (86). In the case of Southern bean mosaic virus additional evidence is available in the identity of the electrophoretic mobility of the virulent agent with that of the characteristic particles (101). The filtration end points of both tobacco necrosis (181) and tomato

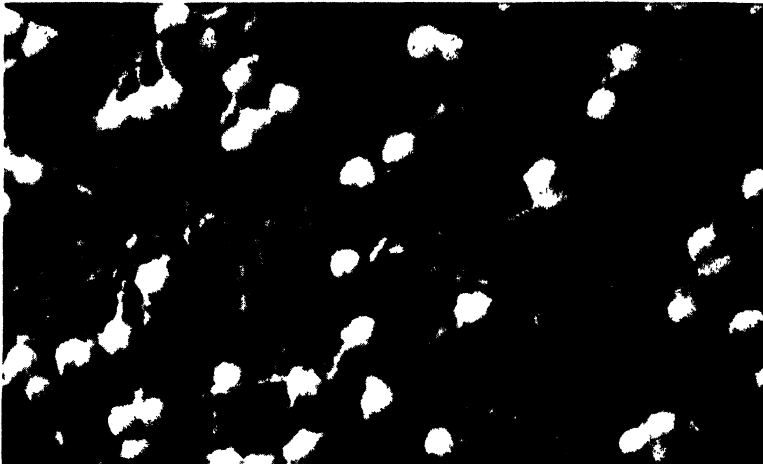


Fig. 5. Electron micrograph of T2 bacteriophage of *Escherichia coli* (173).

bushy stunt viruses (181) indicate particle sizes for the virulent principle compatible with those obtained by physical means for the characteristic particles. It seems fair to regard the evidence in the cases of these several viruses as strongly indicative of identity of virulent agent and characteristic particle, but not as satisfying as the more exhaustive evidence on tobacco mosaic, PR8 influenza A, and vaccinia viruses.

### C. CRYSTALLIZATION OF VIRUSES

Needlelike crystals of tobacco mosaic virus were obtained by Stanley (182) in 1935 by precipitating the virus with ammonium sulfate. A picture of such crystals is shown in Figure 6. It has since

been found that precipitation by numerous other electrolytes and isoelectric precipitation also result in rodlike crystals (21). Cohen (28) observed that heparin, hyaluronic acid and related compounds, and starch were capable of crystallizing tobacco mosaic virus in the form of rodlike entities much like those illustrated in Figure 6. Lauffer has shown that serum albumin is also able to act as a crystallizing agent (94). When the serum albumin and the virus have like charges, rodlike crystals are formed which are composed of virus alone. When the virus and the serum albumin have opposite charges, crystals are formed which are composed of both virus and



Fig. 6. Needlelike crystals of tobacco mosaic virus (184).

serum albumin. Kleczkowski (65) found the tobacco mosaic virus was capable of forming mutual precipitates of this type with several proteins.

Tobacco mosaic virus exhibits many other phenomena closely related to its crystallizability (105). When solutions of the virus are caused to flow, they become birefringent or doubly refracting, as shown in Figure 7. This is due to the orientation of rodlike particles in the flowing stream. Sometimes a liquid crystalline phase will separate from a virus solution. The virus rods orient parallel to each other in small elements of volume called "microtactoids."

Each microtactoid behaves like a crystal with optic axis parallel to the direction of orientation. These microtactoids are somewhat more dense than the virus solution in which they were formed and, therefore settle to the bottom to form a liquid crystalline layer. When electrolytes are added to solutions of the virus, under some conditions, mesomorphic fibers separate (21). These fibers are composed of rodlike virus particles oriented parallel to each other

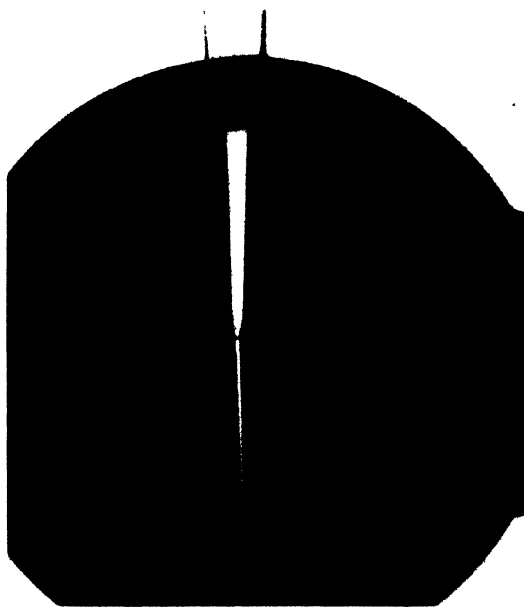


Fig. 7. Doubly refracting stream of tobacco mosaic virus solution flowing between crossed Polaroid plates (105).

but with considerable solvent intervening. In the presence of strong salt solutions, the fibers are caused to shrink laterally and break up into crystals such as those shown in Figure 6. When a solution of the virus is evaporated to dryness under certain conditions, a gel is formed in which the virus rods are lined up parallel to each other. Such gels show strong double refraction and behave like crystals with optic axes parallel to the direction of orientation of the rods (19).





Fig. 8a. Tomato bushy stunt virus crystallized from ammonium sulfate (187).



Fig. 8b. Tomato bushy stunt virus crystallized from sodium heparinate (27)

X-ray diffraction analyses were carried out by Bernal and Fankuchen (19) on crystals of tobacco mosaic virus, on dried gels, and on various liquid crystalline preparations. In all cases it was found that the rodlike particles were oriented parallel to each other with hexagonal symmetry with respect to lateral dimensions but without order in the direction of the long axis. The principal difference between liquid crystalline preparations and dried crystals or dried gels was in the distance between rods in the hexagonal lattice. It was found that this distance was a function of the square root of the reciprocal of the virus concentration. This means that, when water is added to a virus preparation in which the particles are oriented parallel, separation of the particles takes place only in the two dimensions perpendicular to the long axis. The minimum lateral distance between virus rods obtained in three dried preparations was found to be 15.2 m $\mu$ . This figure constitutes a reasonable estimate of the diameter of a dried tobacco mosaic virus rod.

Tomato bushy stunt virus was first obtained in crystalline form by Bawden and Pirie (12) by a salting-out technique. Cohen (27) observed that the virus could also be crystallized with heparin. As shown in Figures 8a-b, the crystals obtained by the salting-out procedure have different form from those obtained by the heparin technique. X-ray analyses were made on the dodecahedra by Bernal, Fankuchen, and Riley (20). Their results were interpreted to indicate that the particles were arranged in a body-centered cubic lattice. Electron micrographs of single layers of bushy stunt virus particles on collodion films show that the virus particles pack in two dimensions in a hexagonal close-packed pattern.

Southern bean mosaic virus was isolated in crystalline form by Price (152). Crystallization can be induced by centrifugation at high speeds (152) and by dialysis against distilled water (132). Pictures of two types of Southern bean mosaic virus crystals are reproduced in Figures 9a-b. Price and Wyckoff (155) studied such crystals with the electron microscope. Air-dried crystals were first coated with a thin layer of metallic gold evaporated from an angle. This layer was then backed up with a thin collodion layer. The two films were removed from the virus crystal by flotation on a water surface. They were then picked up on a microscope screen and photographed. The results showed a rectangular network of particles

in which there was greater separation in one dimension than in the other.

Tobacco necrosis virus has also been crystallized. Unfortunately, there is considerable discrepancy in the literature concerning the

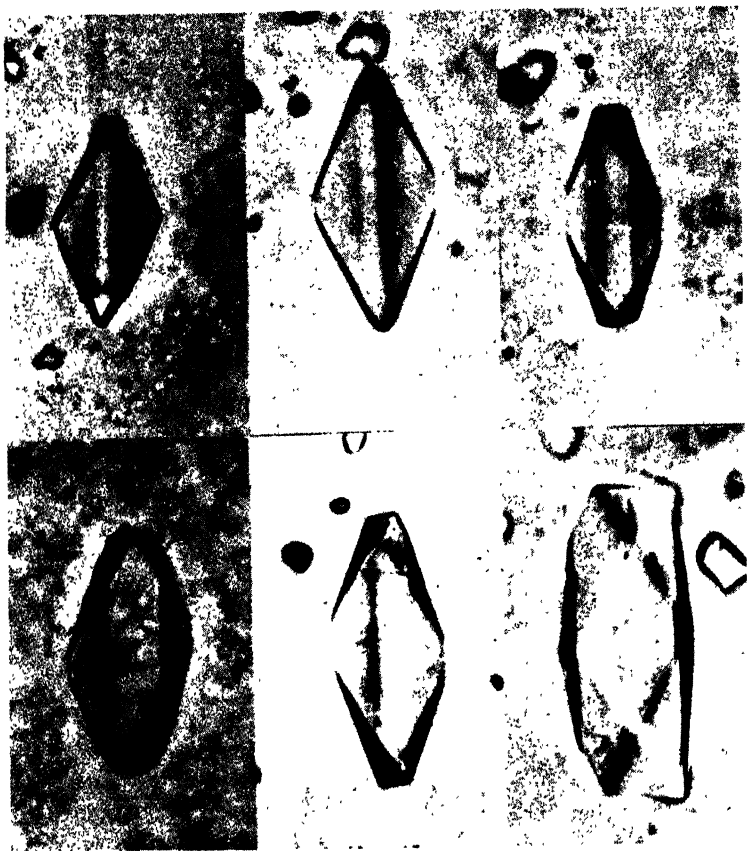


Fig. 9a. Crystals of Southern bean mosaic virus (152).

nature of the virus which causes this disease. The suggestion has been made that there are several viruses which produce symptoms commonly recognized as tobacco necrosis (13). Pictures of crystals obtained by Bawden and Pirie (13) are illustrated in Figure 10.

Crystals of one preparation have been studied by Markham, Smith, and Wyckoff (126) with the electron microscope by the method just described. In this case the particles were found to be arranged in a square network. Further studies by the same technique indicated



Fig. 9b. Crystals of Southern bean mosaic virus (152)

that the most probable structure of the crystal is face-centered cubic (127). This conclusion is not consistent with X-ray evidence published earlier, which indicated a body-centered lattice (31). However, the X-ray studies might have been carried out on a different type of tobacco necrosis crystal.

Markham and Smith (124) reported the crystallization of turnip yellow mosaic virus, crystals of which are shown in Figure 11. Small octahedra were obtained. Cosslett and Markham (29) have reported that preliminary X-ray studies indicate that these crystals have a large unit cell and that the latter may be of the diamond

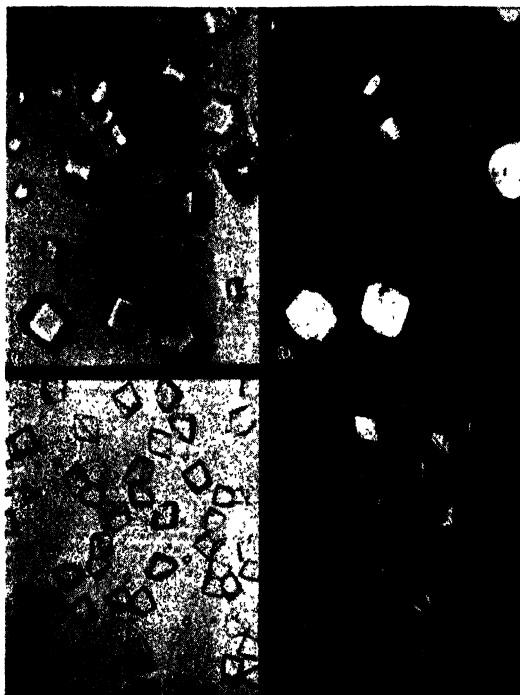


Fig. 10. Crystals of tobacco necrosis virus (12).

type. The same investigators obtained electron micrographs of particles mounted on beryllium films. Some of these show a number of microcrystals which have the appearance of a net formed by hexagonal rings. Each ring has a hole in the middle. This type of arrangement would be consistent with the diamond type of lattice indicated by the X-ray data.

## D. SIZE, SHAPE, AND HYDRATION

For the purpose of simplicity, virus particles and protein molecules can be considered to be approximated by ellipsoids of revolution. It is not implied that any real particle is actually an ellipsoid of revolution; this postulate is made for convenience only, and is useful as long as an ellipsoid of revolution is a reasonable approximation of the actual particle. An ellipsoid of revolution is a solid



Fig. 11. Crystals of turnip yellow mosaic virus (124).

generated by rotating an ellipse about either its long semiaxis or its short semiaxis. Thus, there are two kinds of ellipsoids of revolution: elongated ones and flattened ones. An ellipsoid of revolution can be characterized by its size, expressed in terms of volume or mass, and by its shape. The latter can be expressed mathematically as the ratio of the long to the short semiaxis, with an independent designation as to whether the ellipsoid is of the elongated or of the flattened type. A sphere is a special case in which the ratio of the semiaxes is unity. Thus, the nature of a particle can be approximated by the two parameters, mass or volume and axial ratio, where the former

are measures of size and the latter is a measure of shape. A complication arises by virtue of the possibility that a protein or virus particle might be hydrated when it is in an aqueous medium. If hydration does occur, the mass or volume and possibly even the axial ratio of the hydrated particle will be different from that of the unhydrated or dry particle.

The size of viruses has been a subject of interest for a long time. Crude estimates of size have been provided by the ultrafiltration technique. However, in recent years, more refined data concerning the size and additional information concerning the shape have been obtained. The electron microscope provides an excellent method for determining the size and shape of dried virus preparations. Unfortunately, it provides no evidence concerning the extent of hydration and the ultimate shape of the virus particle in an aqueous medium. The size, shape, and hydration of a particle can be determined, at least in theory, from indirect physical studies such as ultracentrifugation, diffusion, viscosity, double refraction of flow, etc. The application of these methods to the study of viruses has been reviewed in detail elsewhere (90,105). The sedimentation rate, the diffusion constant, and the rotational diffusion constant, which can be determined by stream double refraction studies, depend upon all three of the above attributes of the particle. Intrinsic viscosity is the sole exception; it depends only upon shape and hydration. In theory, if one can make three independent observations each of which is a function of the same three parameters, one can solve for all three parameters. Thus, one should be able to determine the size, shape, and degree of hydration of a particle from sedimentation, diffusion, and viscosity data. Due to inadequacies at the secondary level in the theory or to inability to obtain data of sufficient precision, this possibility has not yet been realized. On the other hand, from any two sets of the four independent measurements, one can obtain size and hydration when shape is known or size and shape when hydration is known.

In the case of tomato bushy stunt virus, Southern bean mosaic virus, and rabbit papilloma virus, the shape of the particle is known to be spherical to a very good degree of approximation. This information has been derived from X-ray diffraction analyses of crystals of tomato bushy stunt virus (20) and from electron micrographs of all three viruses (153,173). Figures 3, 12, and 13 are electron



Fig. 12. Shadowed electron micrograph of tomato bushy stunt virus particle (153).

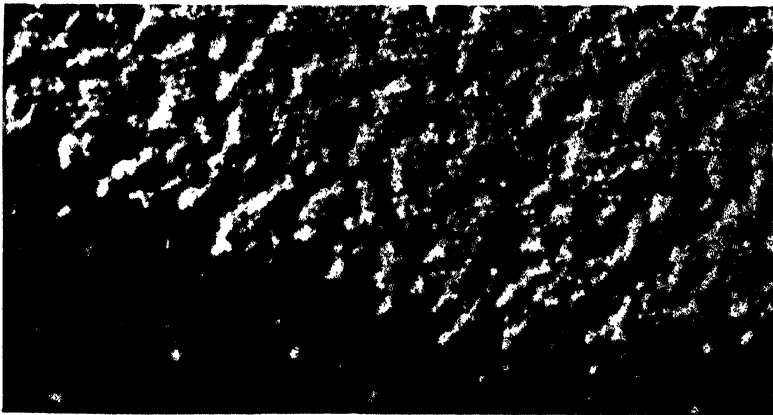


Fig. 13. Shadowed electron micrograph of Southern bean mosaic virus particles (153).

micrographs of rabbit papilloma, tomato bushy stunt, and Southern bean mosaic viruses, respectively. Thus, in all cases it is possible to determine the degree of hydration from either viscosity measure-



ments or from the combination of sedimentation and diffusion measurements. The values obtained are summarized in Table II. The result found for tomato bushy stunt virus is in agreement with a figure obtained from X-ray diffraction analyses of crystals of this virus. Such crystals swell uniformly when brought into contact with moisture. By use of the electron diffraction technique,

TABLE II  
SHAPE, SIZE, AND HYDRATION OF PURIFIED VIRUS MATERIALS\*

Virus	Shape	Hydration, % water by volume	Hydrated dimensions <sup>b</sup>
Tobacco mosaic	Rod, D (104)	15, H (89), I (162)	15 × 250, ABH (89)
	Rod, E (142)	15	15 × 270, ACH (89)
	Rod, F (19)	15	14 × 250, BCH (89)
	Rod, G (140)	15	16 × 280, <sup>c</sup> E (142), F (19)
	Sphere, E (153)	51, A (106) B (134) H (120) 47, F (29), H (120)	37, A (106) B (134) H (120) 31, E (153)
Southern bean mosaic	Sphere, E (153)	49, ABH (131)	31, ABH (131)
		60, C (131)	
		52, IH (131)	32, E (153)
Influenza A	Sphere, E (108)	62, H (108), I (174)	114, A (108)
		62	159, E (108)
Rabbit papil- loma	Sphere, E (173)	58, H (135), I (171)	66, A (135)
		69, C (135)	65, E (170)
		69, ABI (53)	71, ABI (135)

\* Numbers in parentheses refer to bibliography and indicate source of data used in computation. Letters denote type of evidence for each conclusion, according to the following code: A = sedimentation constant, B = diffusion constant, C = intrinsic viscosity, D = double refraction of flow, E = electron micrograph, F = X-ray diffraction pattern, G = light scattering, H = apparent partial specific volume, and I = buoyancy studies with ultracentrifuge.

<sup>b</sup> In millimicrons. Diameter of sphere; thickness (twice short semiaxis) and length (twice long semiaxis) of rods. Each computation is based on hydration value directly to the left of it.

<sup>c</sup> Calculated on the assumption made necessary by the X-ray results of Bernal and Fankuchen that increase in the dimensions of tobacco mosaic virus due to hydration takes place only in directions perpendicular to the long axis.

interparticle distances in the dry state and also in the swollen state were measured. This difference gives an indication of the extent of hydration of the particles in the swollen state. A figure of 47% by volume was obtained, which is in good agreement with the 51%

found by sedimentation and diffusion. In the case of Southern bean mosaic virus a figure of 49% by volume of hydration was obtained from sedimentation and diffusion data. Viscosity measurements yielded a value of 60%.

The problem of interpreting viscosity, sedimentation, and diffusion measurements for particles of either unknown shape or of shape known to deviate from the spherical is very much more involved. Tobacco mosaic virus particles are rod-shaped, as illustrated by Figure 1. In this case, size and shape were determined from three different combinations of sedimentation, diffusion, and viscosity measurements, taken two at a time, on the assumption that the virus particles are not hydrated to any appreciable extent (89). All the results were mutually consistent and agreed reasonably well with the dimensions of 15  $m\mu$  for thickness obtained from X-ray diffraction patterns and 280  $m\mu$  for length obtained from electron micrographs. These dimensions refer to the anhydrous or dry particle. It has been shown that, if tobacco mosaic virus swells at all in water, it swells only in thickness and not in length (19). When this fact is taken into consideration, one can show that sedimentation, diffusion, and viscosity data are not sensitive to hydration. Thus, from such data, one cannot obtain a critical evaluation of the reasonableness of the assumption that the virus particle is not hydrated in solution. It is necessary to have some external criterion to indicate the extent of hydration of tobacco mosaic virus particles.

There are indirect indications that tobacco mosaic virus and other viruses are hydrated to at least a small extent (10,125,146). Most convincing of these is the well-known dispersibility of these agents in aqueous media. Unless there were some chemical compatibility between the virus particles and water molecules, such solution would be impossible. However, such hydration could be limited to a small number of water molecules per virus particle or it could be fairly extensive. No very good way of deciding between these possibilities has been proposed.

A much better quantitative approach to the question of hydration has been under investigation for many years. The rate of sedimentation of a particle in the centrifuge depends upon the difference between the density of the particle and the density of the suspending medium. The particle will fail to sediment when this difference is zero. Thus, by determining the sedimentation rate in

media of different densities one can determine the density of a virus particle, provided the density of the particle does not change when the nature of the medium is changed. The density of anhydrous virus particles can be measured either directly on dried preparations or indirectly by determining the difference between the density of a virus solution of known composition and the density of the solvent. From the densities of the virus in the dry state and in an aqueous medium, it is easy to calculate the amount of water associated with unit amount of virus in the wet state.

The method involving buoyancy measurements with the ultracentrifuge was first used by MacCallum and Oppenheimer (119) in 1922 on vaccinia virus and was later used by Elford and Andrewes (40) in the study of influenza virus. The latter workers observed that in a sugar solution with a density of 1.2 the virus did not

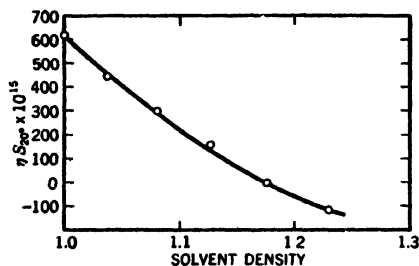


Fig. 14. Sedimentation rate, corrected for viscosity, of PR8 influenza A virus in sucrose solutions of various densities (108).

sediment. They concluded, therefore, that influenza virus has a density of 1.2. More extended studies were made with vaccinia virus by Smadel, Pickels, and Shedlovsky (179), and studies similar to these were carried out on influenza virus in sucrose solutions by Lauffer and Stanley (108), and Sharp *et al.* (174). It was found in all these studies that the rate of sedimentation, after correction for viscosity of the sugar solution, decreased as the density of the sucrose solution increased. However, as illustrated in Figure 14, the data did not fall on a straight line. The theory of sedimentation predicts that sedimentation rate should be directly proportional to the difference between the density of a particle and the density of the medium in which it is suspended. Thus, if the particle does not change density when the composition of the medium is changed, sedimentation rate ought to be a linear function of density of the

medium. One can still evaluate particle density at zero sucrose concentration when the relationship is nonlinear by drawing a tangent to the curve at this sucrose value and finding its intercept with the zero sedimentation rate abscissa. The wet density and, from it, the hydration of Southern bean mosaic virus were determined in this manner (131). As shown in Table II, the value obtained is of the same order of magnitude as values found by other means.

Smadel, Pickels, and Shedlovsky (179) suggested as a possible interpretation for the observed deviation from linearity that water is withdrawn from the virus particle in sucrose solutions of high

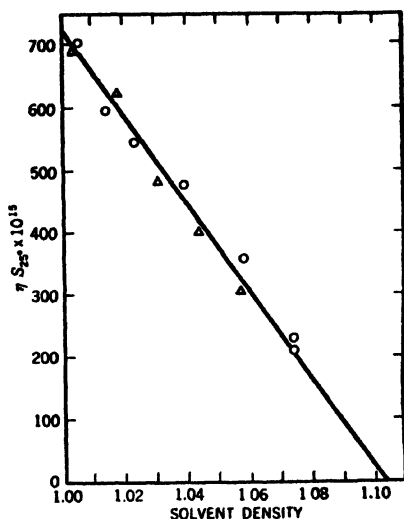
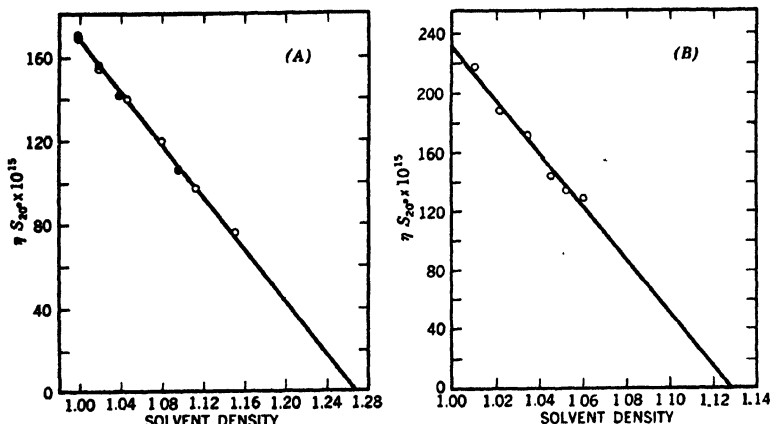


Fig. 15. Sedimentation rate, corrected for viscosity, of PR8 influenza A virus in serum albumin solutions of various densities (174).

density because of the necessarily high osmotic pressures of such solutions. As a result of this suggestion, Sharp *et al.* studied the sedimentation rate of PR8 influenza A virus and of other viruses in bovine serum albumin solutions of various densities (172,174,176). They presented data which fell on straight lines. As illustrated by Figure 15, their data obtained with influenza virus could be extrapolated to zero sedimentation rate at a solvent density of 1.10.

From this they concluded that the density of influenza virus in solution is 1.10. The hydration of influenza virus and also of rabbit papilloma virus were determined from such data (Table II).

This technique of sedimentation in media of various densities has been applied to two strains of tobacco mosaic virus by Schachman and Lauffer (162). As illustrated in Figure 16, it was found that sedimentation rate decreased linearly with medium density both when serum albumin and when sucrose were used to alter density. However, the data obtained with serum albumin extrapolated to zero sedimentation rate at a medium density of 1.13 and the



ness of one-half the effective diameter of the particles used to increase the medium density (162). Forces acting upon a virus particle in the ultracentrifuge, which determine its sedimentation rate, are such that this equivalent layer of water behaves as though it were part of the virus particle. Thus, the density measured is not the density of the virus particle but the mean density of the virus and this equivalent layer of water. The equivalent layer of water is, of course, not part of the particle. When reasonable assumptions are made concerning the effective diameter of sucrose particles and of serum albumin particles, the apparent density obtained in the two sets of sedimentation experiments can be corrected for this effect. When this is done, the two sets of experiments agree reasonably well in indicating that tobacco mosaic virus is hydrated to an extent of about 15% by volume on a wet basis. The error due to this cause in the wet densities of influenza and rabbit papilloma viruses should be less important than that obtained with tobacco mosaic virus because of the much greater ratio of the radii of the virus and serum albumin particles.

The dimensions of tobacco mosaic virus have been calculated from diffusion, sedimentation, viscosity, X-ray, and electron microscope data, on the assumption that the particle is hydrated to the extent of 15% by volume. The results are presented in Table II. In interpreting the data in Table II, it must be remembered that the accuracy of size and shape calculations depends upon the reliability of the information concerning the state of hydration. But the methods used to determine hydration are still in an early stage of development. Therefore, these calculations are meant to illustrate the current state of knowledge rather than to provide a final characterization of the viruses discussed.

Early studies on tobacco mosaic virus indicated that viruses used in various laboratories might differ with respect to particle size. Oster, Knight, and Stanley (141) have recently made electron microscope studies on the contents of hair cells of Turkish tobacco plants infected with tobacco mosaic virus samples from Dahlem, from Rothamsted, and from Princeton. Size distributions of the virus particles of the three samples were indistinguishable. The most frequently occurring length was about 270–280  $m\mu$  in all cases. Knight and Oster (72) investigated eight strains of tobacco mosaic virus in the same manner. These included the ordinary, the mild

(M), the yellow aucuba (YA), the green aucuba (GA), the Holmes rib grass (HR), the J14D1, and the cucumber virus 3 and 4 (CV3 and CV4) strains. The first six showed size distributions with a single mode at about 270–280 m $\mu$ . Some strains seem to be more homogeneous than others. The CV3 and CV4 strains exhibited slightly longer and slightly narrower particles.

The problem of determining the size and shape of tobacco mosaic virus particles was made more difficult by virtue of the fact that this virus is capable of undergoing aggregation. Early sedimentation experiments carried out by Wyckoff (199) showed that some preparations of the virus exhibited more than one boundary in the ultracentrifuge. Double refraction of flow studies carried out by Loring, Lauffer, and Stanley (114) also indicated aggregation when virus preparations were allowed to age. These results were interpreted by Lauffer and Stanley (105) to mean that the virus particles aggregate end to end to form particles of double length. More recent studies (89) with the ultracentrifuge, the electron microscope, and the viscometer bear out the correctness of this conclusion. The question of the aggregation of the rib grass (HR) strain of tobacco mosaic virus has recently been studied by Schachman (161). He observed that the ratio of specific viscosity to concentration of tobacco mosaic virus varied greatly with concentration, particularly for some very old virus preparations. At very low concentrations, he obtained a value for the intrinsic viscosity in good agreement with that obtained by Lauffer for an unaggregated preparation of the virus (89). He was able to interpret his viscosity data in terms of an equilibrium between monomer and dimer. The general shape of his theoretical curve resembled that of the experimental curve showing the relationship between viscosity–concentration ratio and concentration, but there was disagreement at higher concentrations. The existence of reversible aggregation was demonstrated by means of the electron microscope. His data showed that electrolytes play an important role in the process. Chlorine ions seemed to favor aggregation and phosphate ions seemed to favor dissociation.

The use of the methods of ultracentrifugation, diffusion, viscometry, and electron microscopy for the elucidation of the nature of tobacco mosaic virus particles has been subjected to spirited criticism (14,43,146). In general, the criticism follows two patterns.

Some argue that because diffusion rate and sedimentation rate are not independent of concentration and because viscosity is not independent of the rate of flow of the liquid in the viscometer, these methods are incapable of providing reliable estimates of the physical properties of the virus. It must be admitted that these complications make the problem of interpreting data more difficult than it would be otherwise. However, it has been shown that the strong dependence of sedimentation rate upon concentration is correlated with the dependence of solution viscosity upon concentration. When sedimentation rate is corrected for solution viscosity, a figure virtually independent of virus concentration is obtained (89). Whether or not this result has any theoretical significance, at least it provides a very simple and apparently very reliable guide for extrapolating sedimentation and diffusion rates to infinite dilution. Before this relationship was discovered, Frampton (43) showed that it was possible to extrapolate sedimentation and diffusion data to infinite dilution in such ways as to get infinity for the molecular weight by one technique and zero by another. With a reliable principle to guide extrapolation, such an absurdity can be avoided. The question of the anomalous viscosity has been discussed in detail elsewhere (92). As shown in Table II, the indirect methods of physical chemistry give values for the size and shape of tobacco mosaic virus which are mutually consistent and which agree with those obtained by electron microscopy. It can never be stated with certainty that this agreement is not due to chance. However, for three indirect methods to give results, due solely to chance, in agreement with each other and with a fourth direct method would be a phenomenal accident, indeed.

The second point of dispute is whether or not the rods of tobacco mosaic virus,  $15\text{ m}\mu$  thick and  $270\text{ m}\mu$  long, actually are the fundamental virus particle or some aggregate of the fundamental particle. Bawden and Pirie (14) contend that such particles are aggregates of smaller fundamental units which exist in the plant cell. Stanley *et al.*, and Lauffer hold to the opinion that the basic unit which possesses virus activity is the rod  $15 \times 270\text{ m}\mu$  and that the smaller particles frequently observed are degradation products of the virus. It should be emphasized that there is substantial agreement on many important aspects of this question. It is agreed that extremely elongated particles can be formed by the aggregation of tobacco



mosaic virus and that this aggregation can be reversed, at least in part. It is agreed that particles smaller than  $270\text{ m}\mu$  in length can be obtained by various procedures and even that some of these smaller particles can aggregate to form larger particles. Lauffer (87) and Oster and Stanley (142) contend that particles approximately  $270\text{ m}\mu$  long predominate in preparations of the virus, and Bawden and Pirie admit that such particles do, in fact, predominate in many preparations. The sole question remaining is whether these particles,  $15\text{ m}\mu$  thick and  $270\text{ m}\mu$  long, are the fundamental unit of tobacco mosaic virus. This question is an extremely difficult one to resolve experimentally. Experiments carried out with the ultracentrifuge (87) showed that the sedimentation rate of the infectious principle of tobacco mosaic virus agreed within 6% of that of the particles  $270\text{ m}\mu$  long by  $15\text{ m}\mu$  thick. Results of the experiment permitted the unequivocal exclusion of any possibility that the bulk of infectious activity is associated with particles smaller than the  $270\text{ m}\mu$  unit. Yet, it cannot be claimed that the possibility has been eliminated that some small portion of the infectivity is associated with smaller units. Sigurgeirsson and Stanley (177) found that tobacco mosaic virus particles could be broken up into smaller units. These smaller units resembled in some respects those described by Bawden and Pirie (14).

Oster (139) has subjected a preparation of tobacco mosaic virus to sonic vibration at 9000 cycles per second. Activity measurements and electron microscope studies were carried out on original virus and virus irradiated for various times. It was found that the number of particles about  $270$  or  $280\text{ m}\mu$  long decreased exponentially with time. First particles half that long increased in number, then these decreased in number and particles of one-fourth the original length increased in number, etc. Virus activity also decreased exponentially with time. The rate constant for the decrease in particles  $270\text{ m}\mu$  long was of the same order of magnitude, but about 50% greater than that for decrease in activity. This reasonably close agreement is evidence that the  $270\text{ m}\mu$  particles are the bearers of infectivity. Original virus could be caused to aggregate, with a resultant loss of infectivity. The aggregation could be reversed by vigorous stirring, with an accompanying increase in infectivity. Sonically fractured virus could also be aggregated, but without change in infectivity.

Numerous other experiments carried out by other investigators have shown that tobacco mosaic virus can be disintegrated into smaller units, some of which contain nucleic acid and some of which are free of nucleic acid. In all cases thus far reported, including the experiments of Bawden and Pirie, the preparations containing the smaller particles were very much less infectious than those containing the larger particles. If the smaller units are really active virus, one should expect a very much greater infectivity per unit weight of virus material in the smaller fragments. This has never been found to be the case. The results of Bawden and Pirie show progressive decrease in infectivity as the percentage of particles in the smaller size range increase. Electron micrographs published by Crook and Sheffield (30) on various virus preparations submitted by Bawden and Pirie show that all the virus fractions are inhomogeneous. When the relative numbers of rodlike particles as shown by these micrographs are correlated in a rough sort of way with the infectivity obtained by Bawden and Pirie, one can observe a striking parallelism between the occurrence of rods and the existence of infectivity. No experiment has yet been reported which is not consistent with the hypothesis that the fundamental infectious unit of tobacco mosaic virus is a rod 270  $m\mu$  long and 15  $m\mu$  thick. Much evidence consistent with this hypothesis has been made available. It cannot be stated with certainty that an experiment will never be performed which demonstrates the existence of infectivity in smaller particles. However, until such an experiment has been done, it seems unprofitable to complicate the question by postulating the existence of such smaller units. There is no reason to call these smaller particles "virus" until they can be shown to possess high specific infectivity.

## VII. Homogeneity of Virus Preparations

Physical methods of studying viruses are peculiarly well adapted to the problem of determining the homogeneity of the preparation. There are two aspects of the question of homogeneity which are of importance. The first is the association of homogeneity with purity. Homogeneity and purity are not synonymous, but they are related. If a virus preparation can be shown to be homogeneous within the sensitivity of a particular test, and if similar homogeneity is indi-

cated by other tests, one has strong presumptive evidence that the preparation is of a high degree of purity. Evidence of inhomogeneity does not necessarily constitute evidence of impurity of the virus preparation. Several of the methods of determining homogeneity are measures of the size of the virus particles. Two types of inhomogeneity are recognizable: that in which more than one class of particles can be shown to be present, and that in which there exists a single class of particles within which there is an apparently continuous distribution of sizes about a single mean. One can conceive of a perfectly pure suspension which is quite inhomogeneous in the second respect. The importance of purity as a criterion of the identity of a virulent agent with specific particles under study has already been discussed. The importance of purity for biochemical and certain types of biophysical analyses has also been indicated.

The question of homogeneity is also of importance for the evaluation of the concept that viruses are protein molecules. The most severe criterion of molecular nature which can be imposed is that all the particles of a material must be composed of the same number of atoms arranged in the same way. This presupposes absolute homogeneity with respect to the criteria of size, shape, and electric charge. Chemists no longer hold to such a severe criterion for the state of molecular dispersion, but it is adopted in this case in order to lend a substantial margin of safety to any positive conclusion concerning the molecular nature of any particular virus. The concept that protein particles are molecules is the direct outgrowth of the observation that numerous protein preparations fulfill the requirements of absolute homogeneity within the sensitivity of the tests applied. Thus, if any virus meets these standards of homogeneity, precedent exists for making the postulate that such a virus may be a protein molecule.

The physical tools capable of yielding information concerning homogeneity are the ultracentrifuge, the electron microscope, the Tiselius electrophoresis apparatus, and the diffusion cell. Diffusion is the poorest and, for that reason, will not be discussed further. Of the three remaining, the analytical ultracentrifuge is the most sensitive. When a suspension or solution of macromolecules is sedimented in the analytical ultracentrifuge, the particles sediment away from the axis of rotation toward the periphery. The particles in that portion of the solution initially nearest the axis of rotation

will constitute, after the first few moments of sedimentation, a boundary between solution and solvent, and this boundary will move toward the periphery with a rate characteristic of the particles in the solution. Initially, the boundary will be infinitely sharp, but in practice boundaries always become more and more diffuse as sedimentation progresses. This progressive boundary spreading is due in part to diffusion, but it may also be due in part to inhomogeneity with respect to sedimentation rate of the particles in the boundary region. If several classes of particle with appreciably different sedimentation rates are present, more than one boundary will appear. If, on the other hand, the classes of particle have sedimentation rates very close to each other, or if a single class characterized by a distribution in size about a mean value is present, one will then observe a single boundary which spreads to an extent greater than that due to diffusion alone. It is common practice to use some modification of the schlieren optical system for studying the spreading and migration of boundaries. Many viruses give very sharp single boundaries in the ultracentrifuge, and this fact is taken by some as an indication of an unusual degree of homogeneity. It is possible to be misled, however, unless quantitative study is made of the boundary spreading.

The electron microscope as a tool for investigating inhomogeneity is limited by the resolving power of the instrument. Surrounding each image is a zone of uncertainty attributable to this cause. Particularly for the smaller viruses, this sort of uncertainty constitutes an appreciable fraction of the total particle diameter. For that reason, the electron microscope is useful only for determining inhomogeneity with respect to the long dimension of small rodlike virus particles or with respect to the diameters of the larger virus particles.

Tobacco mosaic virus exhibits very sharp boundaries in the ultracentrifuge and this was originally interpreted to mean that the virus is highly homogeneous. More extended study has shown that tobacco mosaic virus is really inhomogeneous in two respects (89,105,200). First of all (as mentioned previously), virus preparations frequently become partially aggregated and show two or sometimes more distinct boundaries in the centrifuge. Preparations of tobacco mosaic virus which exhibit only one boundary in the ultracentrifuge, and that a very sharp one, are also found to be inhomogeneous when

examined carefully (89). Both these types are illustrated in Figure 17. Figure 17A shows a double boundary. Figure 17B shows the boundary spreading of an unaggregated preparation of tobacco mosaic virus and compares it to the theoretical boundary spreading due to diffusion alone. This result was interpreted to indicate that tobacco mosaic virus is inhomogeneous with respect to particle length and that the particular preparation under study was composed of particles characterized by a distribution of lengths about a single mean with a standard deviation of  $\pm 14\%$  of the mean. Electron micrographs on the same virus preparation verified the distribution of particle lengths (89). This is an example of a case

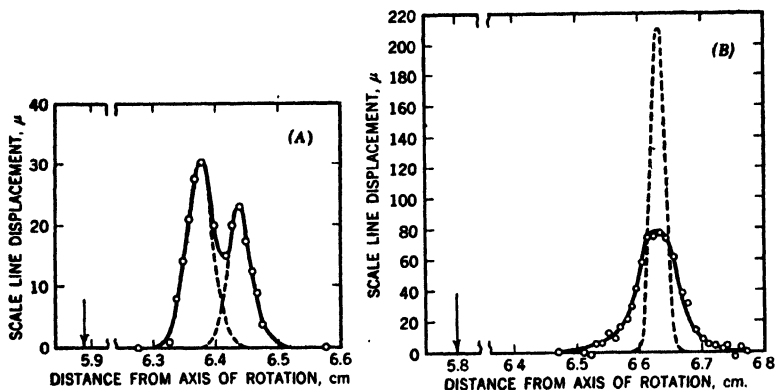


Fig. 17. Graphic representation of tobacco mosaic virus boundaries in the ultracentrifuge (89). (A) Double-boundaried preparation, (B) single-boundaried preparation. Circles are experimental data; broken line in B represents theoretical spreading due to diffusion alone.

in which qualitative consideration leads to the conclusion of homogeneity because of the sharpness of the boundary but in which detailed analysis leads to quite a different conclusion. The inhomogeneity of tobacco mosaic virus might mean that this virus is definitely not molecular in nature. It might also be attributed to the breaking of particles during the purification process. Oster and Stanley (142) have shown that the degree of inhomogeneity is correlated with the extent of handling during purification. Virus preparations obtained by simply expressing fluid from the hair cells of diseased plants onto an electron microscope screen exhibited a much smaller degree of

spread about the mean length than that discussed in the present example. The possibility still exists that tobacco mosaic virus particles in the undisturbed state in the plant are homogeneous with respect to particle length.

Studies carried out with tomato bushy stunt virus in the ultracentrifuge (86) have indicated that the boundary spreading (Fig. 18) in this case can be accounted for quantitatively on the basis of the known diffusion rate of the bushy stunt virus. An attempt was made to evaluate the sensitivity of this criterion as applied to bushy stunt virus. It was shown that the method is sufficiently sensitive to detect inhomogeneity characterized by a distribution of particle sizes with a standard deviation of 1% of the mean. Thus, bushy,

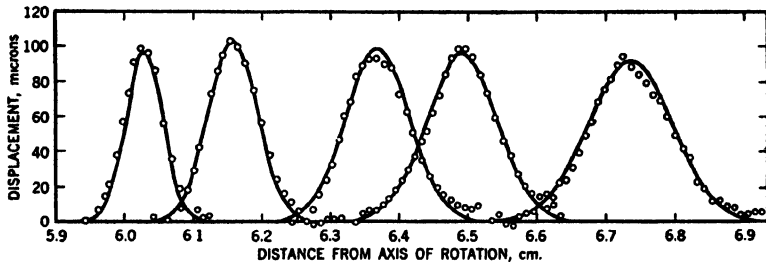


Fig. 18. Theoretical *vs.* actual boundary spreading in sedimentation experiment on bushy stunt virus (86). The circles are experimental points obtained by the Lamm scale method. The smooth curves are theoretical boundary diagrams calculated from the known diffusion constant. The times and the scale-cell distances for the successive boundary curves, beginning at the left are 50 minutes, 2.1 cm.; 85 minutes, 3.1 cm.; 135 minutes, 4.1 cm.; 150 minutes, 4.6 cm.; 220 minutes, 5.6 cm.

stunt virus particles are either homogeneous with respect to sedimentation rate or they are characterized by a size distribution with a standard deviation not greater than 1% of the mean. This constitutes highly sensitive evidence of the homogeneity of bushy stunt particles and permits the interpretation that these virus particles are protein molecules. Quantitative studies of boundaries in the ultracentrifuge have also been made for various strains of influenza virus (108,175) and for vaccinia virus (140). In all cases, a degree of boundary spreading was observed which indicates considerable spread in particle sizes. Electron micrographs agree in indicating

inhomogeneity with respect to particle size (53,108,173,175). Such particles do not meet the criterion for molecular nature.

Electrophoresis in the Tiselius electrophoresis apparatus is capable of indicating homogeneity or inhomogeneity with respect to the criterion of electrochemical constitution. In this case a boundary is established between solution or suspension and solvent and can be observed to migrate in an electric field by the schlieren method or by some other optical method. More than one boundary will appear if particles characterized by more than one distinctly different electrochemical constitution are present. If a single boundary is present it may spread more than in the normal process of diffusion. It is difficult, however, to interpret this in terms of electrochemical inhomogeneity because of several disturbances known to take place at the boundaries. Single boundaries were obtained in the electrophoresis apparatus for tobacco mosaic virus (41), bushy stunt virus (120), and Southern bean mosaic virus (130). A few critically fractionated preparations of PR8 influenza A virus (129) were also found to exhibit single boundaries under a variety of conditions. The electrochemical homogeneity of the tobacco mosaic virus and of the PR8 influenza A virus merely means that the ratio of variation in electrical properties to variation in size is nearly enough constant not to result in electrochemical inhomogeneity. All the preparations of Lee influenza B virus studied by Miller (128) showed more than one boundary, indicative of electrochemical inhomogeneity.

### VIII. Inactivation and Disintegration

For many years it has been recognized that some viruses are more stable than others; in fact, attempts have been made to characterize viruses by an apparent constant known as the temperature of inactivation or the thermal death point. In more recent years, the inactivation reactions of viruses have been recognized as processes which take place according to the laws of chemical kinetics. A stable virus is, therefore, one which becomes inactivated at a slow or negligible rate, and an unstable one is one which becomes inactivated at a more rapid rate. A virus with a high thermal death point is one for which a high temperature is required to obtain a rapid rate of inactivation and, conversely, a virus with a low thermal death point is one which becomes inactivated rapidly at a lower

temperature. Studies have been made on the inactivation of many viruses, but tobacco mosaic virus and PR8 influenza A virus have been studied in greatest detail from the kinetic point of view.

Elementary concepts of chemical kinetics can be used to interpret at least some of the phenomena observed relative to the inactivation and disintegration of viruses. Many virus inactivations seem to be reactions of the first order, that is, reactions in which the number of particles undergoing change per unit of time is directly proportional to the number of unchanged particles remaining at that time. In such reactions one obtains a straight line when logarithm of residual unchanged material is plotted against time. When natural logarithms are used, the slope of this straight line is equal to the specific reaction velocity or the specific reaction rate constant.

Most modern theories of reaction kinetics postulate that ordinary molecules or particles are in equilibrium with activated or high energy molecules or particles and that these activated particles are the ones which actually undergo reaction. Thus, the rate at which a reaction takes place is proportional to the fraction of the molecules or particles in this activated state at any time. Since activated particles are assumed to be in equilibrium with normal particles, this fraction is obviously directly proportional to the equilibrium constant. Therefore, the specific reaction rate is proportional to this equilibrium constant. This concept is very useful because it affords a means of interpreting the variation of reaction rates with temperature, pressure, composition, etc., in terms of the concepts worked out for chemical equilibria. All the evidence at present available indicates that the kinetics of the disintegration and inactivation of viruses can be treated in terms of these concepts.

When tobacco mosaic virus is subjected to the action of heat, high pressures, certain types of radiations, and chemicals such as formaldehyde, urea, sodium dodecylsulfate, acids and bases, the virus is inactivated and the particles are broken down with the loss of nucleic acid into smaller fragments no longer soluble in dilute salt solutions (97,99,107). Price (151) showed that the loss of infectivity by the action of heat followed approximately the course of a first order reaction, and Ross and Stanley (159) and Fischer and Lauffer (42) found that the loss of infectivity in the presence of formaldehyde proceeded according to the law of a first order reaction. The disintegration of the virus particles by the action



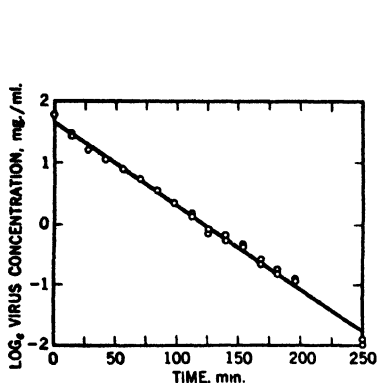


Fig. 19. Natural logarithm of concentration of tobacco mosaic virus protein remaining soluble plotted against time of heating at  $69.8^{\circ}\text{C.}$  in phosphate buffer at  $\text{pH } 7$  (99).

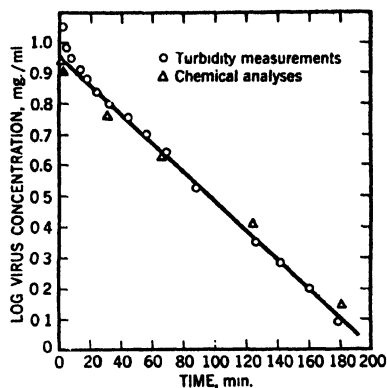


Fig. 20. Logarithm of concentration of tobacco mosaic virus protein remaining undenatured plotted against time of contact at room temperature with  $6\text{ }M$  urea (88).

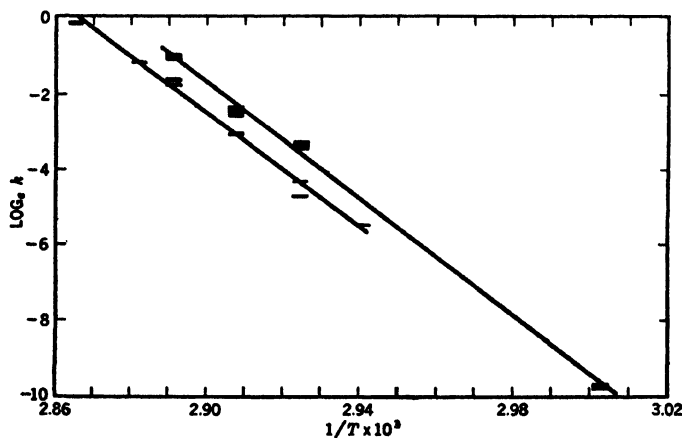


Fig. 21. Natural logarithm of specific reaction rate ( $k$ ) for denaturation of tobacco mosaic virus protein plotted against reciprocal of absolute temperature (99). Upper line: initial virus concentration of 3 mg. per milliliter; lower line: initial virus concentration of 6 mg. per milliliter.

of heat (99) and also by the action of urea (88) have been shown to be first order processes, as illustrated in Figures 19 and 20. When the natural logarithm of the specific reaction rate for the disintegration of tobacco mosaic virus by heat is plotted against the reciprocal of the absolute temperature, a straight line relationship is obtained, as illustrated in Figure 21. This situation is exactly analogous to that obtained for an equilibrium constant. In the case of the equilibrium constant, the slope of the straight line is equal to the product of the gas constant and the heat of the reaction under study. Applying the analogy to the disintegration of tobacco mosaic virus, the slope of the line is proportional to the heat absorbed in changing a mole of tobacco mosaic virus from the normal state into the activated state. This is approximately the same as the energy of activation. A value of 153,000 cal. per mole was obtained for this reaction.

The disintegration of tobacco mosaic virus in the presence of concentrated urea was also found to be a first order process with respect to virus (88). In this case a very interesting temperature anomaly was observed. As illustrated in Figure 22, it was found that the disintegration of tobacco mosaic virus in the presence of strong solutions of urea proceeded most slowly at about room temperature and more rapidly at both higher and lower temperatures. This unusual behavior was confirmed for several viruses. It can be explained on the basis of the assumption that tobacco mosaic virus becomes activated in the presence of urea by reacting in a reversible manner with the urea. To explain the unusual dependence upon temperature, it is necessary to postulate that two simultaneous routes are available for the destruction of the virus, one of which involves an exothermic activation process and the other of which involves an endothermic activation process. The specific reaction rate of the disintegration in the presence of urea and also of the disintegration at elevated temperatures was found to depend upon the initial virus concentration. In general, the lower the initial virus concentration, the more rapidly it disintegrates. In spite of this, the virus concentration decreases exponentially with time, as it should in a first order process. The effect of initial concentration on rate of disintegration in the presence of urea is illustrated in Figure 23. Many viruses exhibit similar phenomena. In general, viruses are more

stable to inactivation in concentrated solution or even in the presence of extraneous proteins. As a matter of fact, it has long been recognized that most proteins become denatured more readily in dilute solutions than in concentrated solutions.

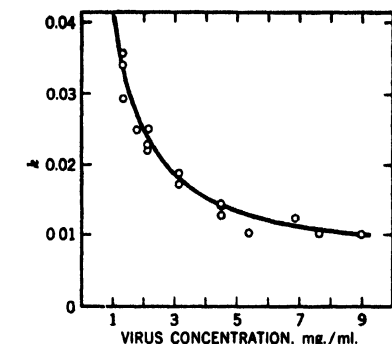
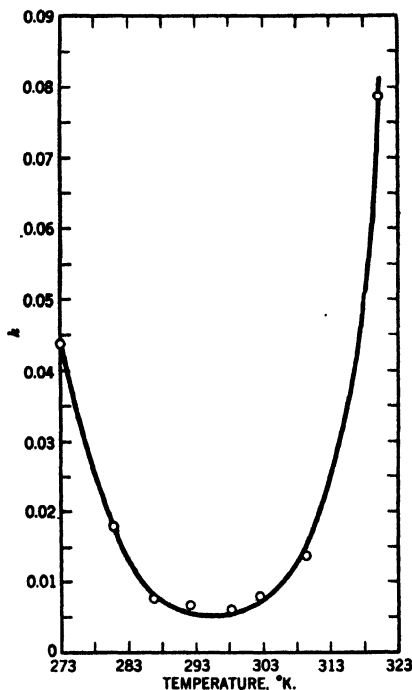


Fig. 23. Specific reaction velocity ( $k$ ) for denaturation of tobacco mosaic virus protein in 6  $M$  urea plotted against initial virus concentrations (88).

Fig. 22. Specific reaction velocity ( $k$ ) for denaturation of tobacco mosaic virus protein in 6  $M$  urea plotted against absolute temperature (88).

The disintegration of tobacco mosaic virus at extremely high pressures has also been investigated (97). At a pressure of 5000 kg. per square centimeter, the virus is broken down at 30°C. into smaller fragments much more rapidly than at normal atmospheric pressure. This can be interpreted to mean that the process of activation involved in this particular reaction involves a decrease in volume. Just as the change in the equilibrium constant of a chemical reaction with pressure can be related to the change in molar volume of the reaction, the change in reaction rate with pressure can be related to a decrease in volume in the activation process. This

decrease was about  $97,000 \text{ cm}^3$  per mole. At intermediate pressures, however, the rate of destruction of tobacco mosaic virus was found by Johnson and collaborators (64) to be slower than at room temperature. For this process it was necessary to postulate an increase in volume when a virus particle goes from the normal state to the activated state. A value of  $100 \text{ cm}^3$  per mole was obtained.

Tobacco mosaic virus can also be inactivated by means of irradiation (109). In this case the extent of inactivation is related in an exponential way to the total amount of radiation. It has been shown

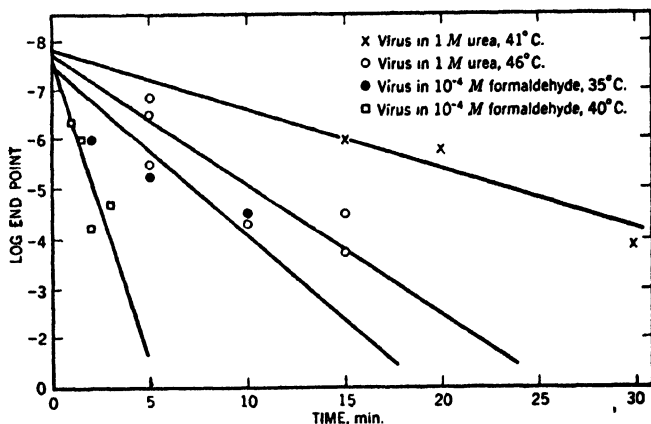


Fig. 24. Logarithm of 50% infectivity end point of PR8 influenza A plotted against time of heating (102).

that the rate of inactivation by radiation can be interpreted as being proportional to the volume of that portion of a virus particle which is sensitive to radiation. In the case of tobacco mosaic virus the radiation sensitive volume of a particle is considerably less than the volume obtained by indirect physical means and by electron microscopy.

The inactivation of the PR8 strain of influenza A virus has also been subjected to considerable study (95,102,103). It was found, as illustrated in Figure 24, that the destruction of infectivity of this virus by heat and the destruction in the presence of urea and formaldehyde proceed as first order processes. The effects of such variables as temperature, pH, urea concentration, and formaldehyde

concentration have been studied one at a time. Results are shown in Figures 25 to 28. In the absence of any extraneous chemical agents,

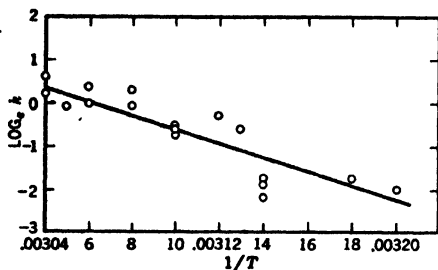


Fig. 25. Natural logarithm of specific reaction rate ( $k$ ) for destruction of PR8 influenza A virus infectivity plotted against reciprocal of absolute temperature (95).

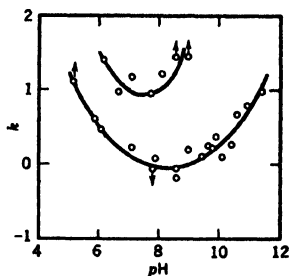


Fig. 26. Specific reaction rate ( $k$ ) for destruction of PR8 influenza A virus infectivity vs. pH (95). Upper curve 54°C., lower curve 46°C.

the destruction was found to have an energy of activation of 34,000 cal. per mole. The reaction proceeded at a minimum rate at about

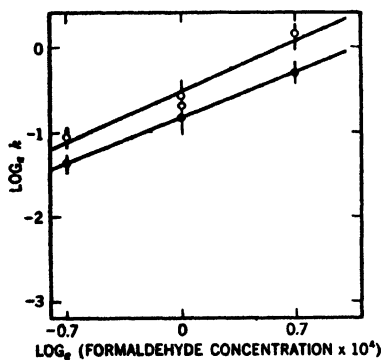


Fig. 27. Natural logarithm of specific reaction rate ( $k$ ) for PR8 influenza A virus infectivity destruction plotted against natural logarithm of formaldehyde concentration (102). Upper curve, pH 7.0, 30°C. Lower curve, pH 8.0, 25°C.

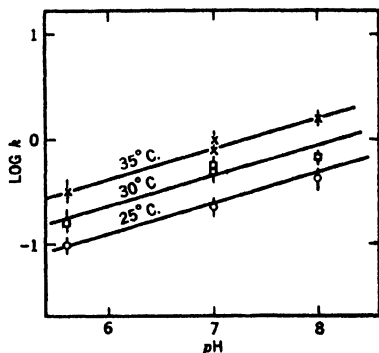
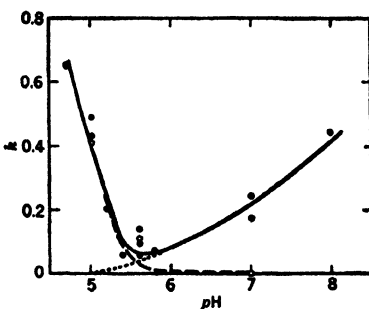


Fig. 27A. Logarithm of specific reaction rate ( $k$ ) for destruction of PR8 influenza A virus infectivity plotted against pH of medium (103).

pH 8. In the presence of 1 molal urea, the energy of activation was about 48,000 cal. per mole, and in the presence of 2 molal urea,

about 52,000 cal. per mole. The energy of activation for the destruction of influenza virus activity in the presence of formaldehyde at a concentration of  $10^{-4}$  g. per milliliter was about 20,000 cal. per mole at pH 5.6, 7.0, and 8.0. The reaction rate, however, at constant temperature and constant formaldehyde concentration was found

Fig. 28. Reaction rates at 25°C. and  $10^{-4}$  g. formaldehyde per milliliter plotted as ordinates against pH for range from 4.7 to 8.0: Broken line represents rates in absence of formaldehyde; dotted line represents rates in presence of formaldehyde; solid line represents sum of the other two (103).



to decrease as pH decreased over the range from pH 8.0 to 5.6, and to increase as pH decreased at pH values between 5.6 and 4.7. Thus, the minimum rate occurs at pH 5.6. This is in marked contrast with the situation obtained in the absence of formaldehyde.

The kinetics of the destruction of the red blood cell agglutinating activity of PR8 influenza A virus has also been studied (96, 164,165). In this case, adherence to the law of a first order process was not obtained. On the contrary, straight line relationships were obtained when the reciprocal of the square root of residual red blood cell agglutinating

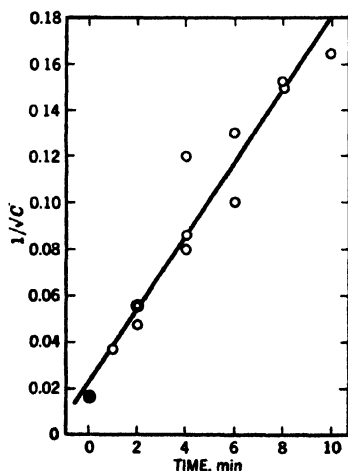


Fig. 29. Reciprocal of square root of red blood cell agglutination activity ( $C$ ) of PR8 influenza A virus plotted against time (96).

activity was plotted against time of treatment, as illustrated in Figure 29. This is a reaction of the  $3/2$  order. It is believed that this type of rate process can be attributed to inhomogeneity of the virus with respect to the criterion of the stability of the red blood cell agglutination principle. The  $3/2$  order law, however, does serve as a means of evaluating a reaction rate constant characteristic of the reaction. This rate constant varies with temperature in the same manner as does the rate constant for the

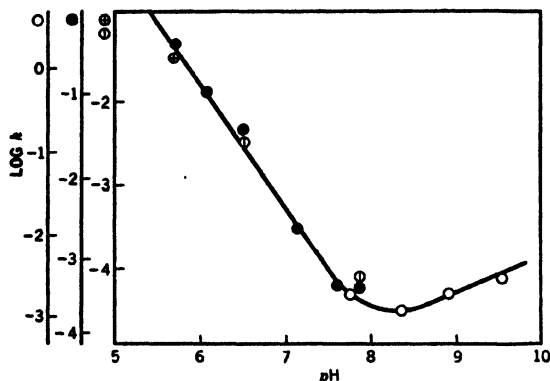


Fig. 30. Logarithm of specific reaction rate ( $k$ ) for destruction of red blood cell agglutination activity of PR8 influenza A virus (at  $55^{\circ}\text{C}.$ ) plotted *vs.*  $\text{pH}$  (165).

destruction of the infectivity. The energy of activation for this process was found to be about 110,000 cal. per mole. The rate of destruction of the red blood cell agglutination principle at constant temperature was found to vary with  $\text{pH}$  and to have a minimum at about  $\text{pH}$  8 (Figure 30). It was also found to increase upon the addition of urea.

## IX. Chemical Composition

One of the major concerns of investigators in the field of virology is the structure of virus particles. It has already been shown that biophysical techniques are capable of resolving questions of gross

morphology. These techniques are also capable of throwing some light upon the gross structure of degradation products. However, to understand the fine structure, it is necessary to have detailed information concerning the chemical composition of the material and concerning the way in which the components are arranged. Thus, before the structure of a virus can be understood, chemical analyses must be available. Extensive chemical analyses have been carried out on vaccinia virus, T2 bacteriophage of *Escherichia coli*, PR8 influenza A and Lee influenza B viruses, and various strains of tobacco mosaic virus. Analyses of vaccinia virus and the T2 bacteriophage, both of which have been reviewed previously (15,28,55, 178), were confined to determination of the classes of compound present. Both viruses were shown to be complex entities. The development of biological assay methods for amino acids has made possible analyses of the amino acid composition of the nucleoproteins of several strains of influenza and of tobacco mosaic viruses.

Biochemical analyses provide a key to the structure of a virus only when performed upon relatively pure materials. Some of the difficulties relative to the purity of influenza virus preparations have already been discussed. A highly viscous contaminant (108,175), which causes sedimentation rate to vary strongly with concentration, has been found in preparations obtained both by differential centrifugation as well as by chicken red blood cell agglutination. Electrophoresis experiments (128,129) have indicated inhomogeneity of both PR8 influenza A and Lee influenza B preparations obtained by ordinary techniques. The immunochemical studies of Knight (66,67) show that antigens characteristic of the host are present in influenza virus preparations. Those facts must be borne in mind in interpreting the extensive chemical analyses carried out on influenza virus preparations.

Preparations of PR8 influenza A and Lee influenza B viruses have been subjected to chemical analyses by Taylor and by Knight. Analyses on the nucleoprotein of PR8 influenza A virus first indicated that the nucleic acid was of the desoxyribose type (190). However, Knight (68) was able to demonstrate both ribose and desoxyribose. Due to unavailability of a satisfactory quantitative method of measuring desoxyribose, no better than a rough estimate could be obtained of the distribution of nucleic acid between the



ribose and desoxyribose types. Neither is the question of predominance of ribose and desoxyribose in Lee influenza B virus readily resolved. The Lee influenza B virus preparation obtained by Taylor (190) seemed to contain mostly desoxyribonucleic acid, while that obtained by Knight was predominantly of the ribose type. The simultaneous existence of the two types of nucleic acid in close association has hitherto been demonstrated in the case of some of the bacteria but not for any of the viruses.

In addition to nucleoprotein, influenza virus preparations have been found to contain a lipid component, a protein component related serologically to the constituents of the host, and a polysaccharide. Taylor found that the lipid is extractable and Knight found that the nucleic acid is less readily split off than that of other viruses. In addition to the carbohydrate of the nucleic acid, there has been found a polysaccharide of mannose and galactose. Drastic procedures were found necessary to effect extraction and separation of the individual components. Accordingly, it is difficult to determine whether these components are essential to activity. There is experimental evidence on this point (71,190) but it cannot be interpreted without reservation. Taylor (190) found that petroleum ether extraction removed little lipid and that some activity was simultaneously lost. He found distinguishable differences: with respect to total lipides and cholesterol, the B preparation had a lower concentration than the other two types; with respect to phospholipides the swine preparation had a lower concentration than either of the other two preparations.

Studies on the carbohydrate components of Lee influenza B virus preparations were carried out by Taylor. The amount of carbohydrate found was greater than required for the nucleic acid as indicated by the phosphorus content. Acid hydrolysis was required before a Molisch test could be obtained. In addition to the desoxyribose, various tests indicated a polysaccharide of either mannose-galactose or glucose-galactose type. Knight investigated preparations of both the PR8 influenza A and also preparations of the Lee influenza B viruses. He concluded from his studies that the carbohydrate was of the mannose-galactose type. Since he was unable to account for all the nitrogen in one of the carbohydrate fractions as protein, Knight concluded that glucosamine must be present. This glucosamine appeared to be associated with the pro-

tein of allantoic origin. Removal of it in a preparation increased the chicken cell agglutinating activity. Thus, in addition to the finding of a polysaccharide in the influenza virus particle previously reported by Taylor, Knight reported the presence of glucosamine. These carbohydrates he also found to be present in the normal

TABLE III  
AMINO ACID RESIDUE CONTENT OF VIRUSES<sup>a</sup>

Amino acid	TMV	M	J14D1	GA	YA	HR	CV3	CV4	PR8	Lee	Allantoic particle
Alanine	4.1	4.1	3.8	4.1	4.1	5.1	—	4.9	2.0	2.1	—
Arginine	8.8	8.9	8.9	9.9	10.0	8.9	8.3	8.3	4.5	3.6	3.5
Aspartic acid	11.7	11.7	11.6	11.8	11.9	10.9	—	11.3	6.4	6.3	5.4
Cysteine	0.6	0.6	0.5	0.5	0.5	0.6	—	—	—	—	—
Glutamic acid	9.9	10.1	9.1	10.1	9.9	13.6	5.6	5.7	6.8	5.4	5.3
Glycine	1.4	1.3	1.4	1.4	1.4	1.0	0.9	1.1	1.9	2.2	1.4
Histidine	—	—	—	—	—	0.6	—	—	1.2	1.3	0.7
Isoleucine	5.7	5.8	5.7	4.9	4.9	5.1	4.7	4.0	3.5	3.6	2.8
Leucine	8.0	8.0	8.1	7.9	8.1	7.7	8.0	8.1	4.6	4.7	3.7
Lysine	1.3	1.3	1.7	1.3	1.3	1.3	2.2	2.1	3.2	4.1	2.2
Methionine	—	—	—	—	—	1.9	—	—	2.0	1.8	1.0
Phenylalanine	7.5	7.5	7.5	7.4	7.5	4.8	8.8	8.7	3.3	3.0	3.2
Proline	4.9	5.0	4.6	4.9	4.8	4.6	—	4.8	2.2	2.3	2.4
Serine	5.9	5.8	5.6	5.8	5.9	4.7	7.7	7.8	1.8	1.8	1.7
Threonine	8.4	8.6	8.5	8.8	8.6	6.9	5.9	5.9	3.1	3.4	3.2
Tryptophan	1.9	2.0	2.0	1.9	1.9	1.3	0.5	0.5	1.0	0.6	0.6
Tyrosine	3.4	3.4	3.5	3.3	3.3	6.1	3.4	3.3	2.8	1.9	2.0
Valine	7.8	7.6	7.5	7.4	7.7	5.2	7.4	7.5	2.9	2.7	2.7
Nucleic acid	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
<i>Total</i>	<i>96.3</i>	<i>96.7</i>	<i>95.0</i>	<i>96.4</i>	<i>96.8</i>	<i>95.3</i>	<i>68.4</i>	<i>89.0</i>	<i>58.2</i>	<i>55.8</i>	<i>46.8</i>

<sup>a</sup> Various strains of tobacco mosaic virus are designated as follows: TMV = regular, M = mild, J<sub>14</sub>D<sub>1</sub> = lethal variant isolated by Jensen, GA = green aucuba, YA = yellow aucuba, HR = Holmes rib grass, CV3 and CV4 = cucumber virus 3 and 4, respectively. The last three columns refer to influenza virus and a constituent of normal allantoic fluid.

proteins of chick embryo allantoic fluid. For one preparation of Lee virus carbohydrate the polysaccharide was reported to consist of glucosamine, mannose, and galactose in the proportion 2/1/1 and, for a preparation originating from PR8 virus, the proportion found was 1/1/1. Examination of the serological behavior of the polysaccharide fraction showed it to give precipitates with antisera to the

intact PR8 influenza virus and to the sedimentable protein of normal allantoic fluid.

Detailed amino acid analyses on preparations of the PR8 and Lee strains were made by Knight (69). The results are shown in Table III. The amino acid residue composition totals about 50 to 60% of the material present. The balance is presumably composed of the other constituents just discussed. The analytical results are

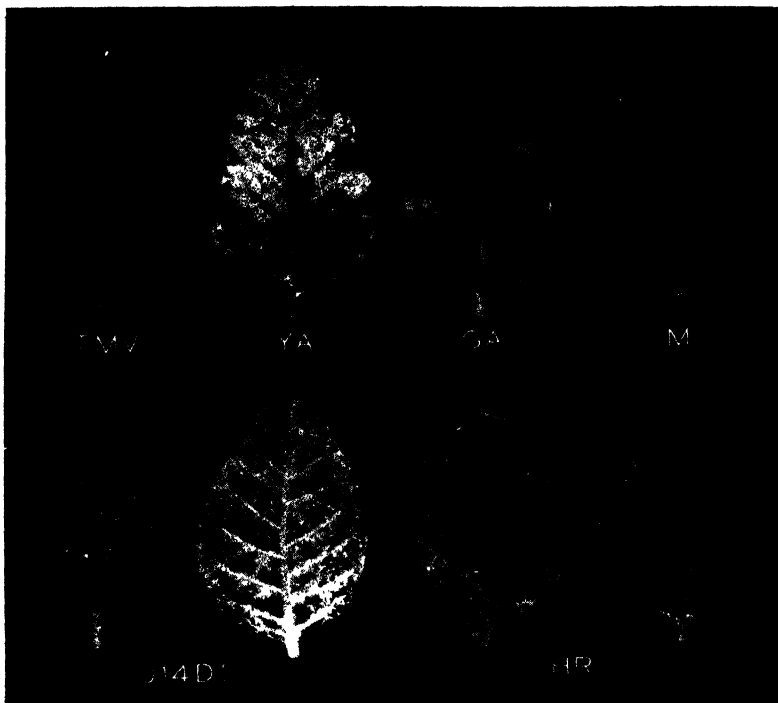


Fig. 31. Symptoms of six strains of tobacco mosaic virus on leaves of Turkish tobacco (72).

consistent for various preparations of the same virus. Definite differences between the PR8 and Lee preparations are indicated by these studies.

The situation with tobacco mosaic virus is more favorable. There is little reason to doubt that preparations of a high degree of chemical purity can be obtained readily. Tobacco mosaic virus has been

shown to be composed entirely or almost entirely of nucleoprotein. The nucleic acid portion of the particle has been analyzed by Loring. It is of the ribose type but differs slightly from typical yeast nucleic acid. Early chemical analyses of the amino acid composition of this virus were carried out by Ross and Stanley and by others. These early results have been reviewed previously (93). Knight has carried out amino acid analyses on preparations of the ordinary (TMV), the mild (M), the J14D1, the green aucuba (GA), the yellow aucuba (YA), the Holmes rib grass (HR), the cucumber virus 3 (CV3), and the cucumber virus 4 (CV4) strains of tobacco mosaic virus (70). Results are shown in Table III. The amounts of the amino acids found are sufficient to account for 95% of the particle in all cases except the two cucumber strains. The rib grass strain contains methionine and histidine, which seem to be absent from the others. Leaves from plants diseased with some of these strains are shown in Figure 31.

Closely related strains show the closest resemblance in composition. The regular and the mild tobacco mosaic viruses differ very little. The yellow and green aucuba strains are closely similar, whereas rib grass virus, which gives a drastically different symptom pattern, is decidedly different in composition. Strain J14D1, a lethal variant of a strain of tobacco mosaic virus, differs significantly in the amount of only two amino acids. It can be cited as an instance of a change or mutation, measurable by the microbiological amino acid analyses, yet not drastic in extent, which results in an overpowering increase in the lethal effect of the virus toward Turkish tobacco.

The chemical composition of the virus protein was shown by Gaw and Stanley (51) to be a characteristic of the virus strain and not of the host plant. Rib grass and ordinary tobacco mosaic viruses were grown in phlox and in Turkish tobacco plants. When preparations of the two virus proteins, each isolated from both host plants, were analyzed for amino acids, values for ordinary tobacco mosaic virus preparations from the two hosts were identical, for the rib grass strain, practically so. Linear relationships between concentration and specific viscosity show the tobacco mosaic virus preparations from the two hosts to be indistinguishable; a perceptible difference in the rib grass preparations may be due to a variation in the state of aggregation (161). The phenylalanine and tryptophan

values reported are subject to recalculation (70,72). This, however, does not affect the relationships in these values.

Based on the values given by Knight (69,70), the percentage composition for amino acid peptide residues in the dry proteins of influenza and tobacco mosaic virus are given in Table III. The nucleic acid contents of these viruses are approximately 5% (68,113,163).

## X. Conclusions

After the crystallization of tobacco mosaic virus by Stanley (182) in 1935, it seemed that knowledge regarding the nature of viruses might be rapidly accumulated. It appeared possible that viruses might be protein molecules only a little more complex than the enzymes or other proteins that occur normally in plants and animals. A full understanding of the fundamental nature of viruses seemed not far distant. Actually, much has been accomplished, as is indicated in preceding paragraphs. However, we are still far from a complete understanding. One of the reasons for this is that as yet we have devised no methods for seeing how viruses reproduce or even for studying their reproduction quantitatively. Another reason is that relatively few viruses have so far been obtained in a state of demonstrated high purity.

In characterizing viruses it is not sufficient to know their size, shape, and degree of hydration. The physical aspects of virus particles, important as they may be, represent only one facet of their fundamental nature. It is important to know also the biochemical structure of the particles and something of their biological activities. Chemical analyses of purified preparations can be no more accurate than the state of purity of the preparation. Knowledge of the physical characteristics of virus particles aids in obtaining purified preparations, but, as pointed out above, it is possible to determine the size and shape from studies on impure preparations. A physical picture of a virus also helps in identifying it in cellular exudates of diseased plants or animals, as has been done on numerous occasions by use of the electron microscope. However, it is not certain that the forms seen in such exudates are the only forms in which the virus exists in its host. The search for intermediate forms must go on until the answer to how viruses reproduce is known.

The size and shape of several viruses have been established fairly accurately. It is becoming apparent that viruses vary in size and shape as much as bacteria. Many of the plant viruses and some of the animal viruses are approximately spherical, others are elongated rods. Elementary bodies of vaccinia are short cylinders, apparently inhomogeneous internally. The T2 bacteriophage of *Escherichia coli* is tadpole-shaped, having a roughly spherical head and an elongated tail. The largest virus has a volume of the order of magnitude of about 10,000 times that of the smallest. Yet, despite these physical diversities, viruses show remarkable similarities in their biological effects.

Viruses also show pronounced dissimilarities in chemical composition. All of the plant viruses so far studied seem to consist of nucleoprotein, as do some of the animal viruses. Others, like vaccinia virus, contain fat and carbohydrate as well as protein. The exact chemical composition of most viruses is uncertain because of the uncertainty in purity of the preparations that have been analyzed. Fortunately, it has been possible to obtain tobacco mosaic virus in a highly purified state. The studies of Knight have therefore yielded fairly exact information about its chemical composition. The virus consists of about sixteen to eighteen amino acids, depending upon the strain. Strains differ significantly with respect to their amino acid composition. There has been as yet no attempt to correlate amino acid composition with specific biological activity. It would be of interest to know, for example, whether the ability to produce yellowing in a host can be correlated with presence or absence of some specific amino acid. Studies along these lines might yield information about the way in which viruses induce characteristic responses in their hosts.

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# THE MECHANISM OF FERTILIZATION IN METAZOA

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*The nature of the inhibition that causes the need of fertilization is a most fundamental problem.*

*It would appear that the presence of this colloid substance in the cortex is an inhibition to the maturation of the egg.*

Fr. R. Lillie, *J. Morphol.*, 22, 361 (1911)

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## I. Introduction

Sexual differentiation is a fundamental characteristic of animals and plants, including the unicellular organisms. When sexual differentiation is not found, it is very likely that secondary suppression has taken place. In recent years the problem of sexual differentiation has been most exhaustively discussed by Hartmann (88,89).

The sexual cells are called gametes. In unicellular organisms male and female gametes may be indistinguishable, or very pronounced differences may exist. In the former case, however, the gametes differ with respect to certain chemical properties. Strong

differentiation between the male and female gametes prevails in metazoa and metaphyta. Fertilization involves the union of two sexually differentiated gametes and their nuclei. The latter have the reduced or haploid number of chromosomes. Reduction is brought about through two "meiotic divisions." In an early phase of these divisions a pairing of homologous chromosomes occurs; exchange of parts may take place between the paired chromosomes, a phenomenon called crossing over. A kind of exchange has recently been demonstrated even in bacteria (134) and viruses (105,169). Some kind of sexual processes probably occur even in these levels of organization. Through the union of the gamete nuclei in fertilization the diploid number of the chromosomes is re-established. The process of fertilization preceded by crossing over involves a recombination of the genes localized in the chromosomes. In this way the sexual processes promote variability, which ever since Weissmann has been regarded as the essential biological significance of fertilization. This view, although it does not account for the so-called autogamy which occurs in certain protozoa, is justified by an enormous amount of data from genetics.

The problem of fertilization has, however, not only a genetic but also a physiological-biochemical aspect. It remains to be explained by what mechanism the gametes meet, how they fuse, and above all what physiological role fertilization plays in the life cycle. Maupas, through experiments on ciliate Infusoria, arrived at the conclusion that a state of aging occurs after many generations of asexual reproduction. This state is abolished by fertilization. It has since been shown, however, that sexual processes need not necessarily occur in the life cycle of protozoa. Under favorable conditions of culture no aging and no fertilization take place. Thus it seems that there is no general connection between reproduction and sexuality in protozoa. For literature and detailed discussion see Hartmann (88,89) and Wilson (302). In metazoa and metaphyta, on the other hand, such a connection has been established. In general, the eggs develop only when fertilized. The process which causes the development of these eggs is called "activation."

After the oogonial divisions have taken place the oocytes undergo a period of growth. Sooner or later following conclusion of this period a block is established which inhibits further division of the

cell and probably causes a general inertness of the anabolic processes (see 12,53,137). In some animals the block inhibits even the meiosis, in others this process may proceed until a certain definite stage (metaphase of first or second meiotic division). Finally, in some metazoa (sea urchins) and in metaphyta, the block operates only after completion of meiosis. For literature and further details see (123, p. 152; 302, p. 595).

The block has a fundamental adaptive value in that it maintains the female gamete in a relatively constant state until fertilization is brought about (see Dalcq, Pasteels, and Brachet, 53). The block established in the oocytes or the mature unfertilized egg is thus of great significance from the biological point of view but it also constitutes a major problem in the biochemical approach to the activation process.

In a number of metazoa or metaphyta parthenogenetic development of the egg occurs constantly or as an alternative to bisexual reproduction. No block is established in these cases or the block is removed without fertilization. It is of interest that in some protozoa and algae both the male and the female gametes are capable of parthenogenetic development (see 88, p. 2). The spermatozoa of the metazoa are so highly specialized that one would not expect them to be capable of development. Loeb and Bancroft have, however, kept spermatozoa of the fowl in a nutritive medium under sterile conditions in order to find out whether development phenomena would occur. The result was negative (see 163, p. 304).

The discussion in this article will be restricted mainly to activation of the eggs of metazoa. It must be realized, however, that our knowledge in this field is still very incomplete. Any attempts to form general conclusions must therefore remain tentative. Certain animals have offered more favorable conditions for research than others and have therefore been more thoroughly studied. In the following the evidence revealed by studies on sea urchin eggs will predominate. No claim is made that references to the literature are complete. This article had already been outlined when the excellent recent review by Tyler (277) on "Fertilization and Immunity" became available to the writer. A certain overlapping of the content of the two articles cannot be avoided, but in many respects they are complementary.

## II. Dependence of Fertilizability on Physiological State of the Sex Cells

Probably the fertilizability of the female sex cell always presents a more or less pronounced optimum depending on the stage of development of the cell. This was demonstrated by Delage (55) in his classical experiment on starfish eggs. Nonnucleated fragments of primary oocytes with intact germinal vesicles were not fertilizable. After breakdown of the germinal vesicle had begun fertilizability of the nonnucleated fragment ensued but its optimum was attained only after the complete breakdown of the germinal vesicle. After the separation of the second polar body fertilizability declined. Delage inferred that "cytoplasmic maturation" is brought about by the penetration of nuclear material into the cytoplasm. Similar conditions were found by Delage and other authors in the eggs of certain annelids and molluscs. For literature and discussion, see Costello (50). In the annelid *Nereis* even the primary oocyte with intact germinal vesicle is fertilizable. Costello subjected these cells to centrifugal force 66,000 times gravity and divided the elongated oocytes into two parts with a needle. Subsequent to insemination only the nucleated part developed. Penetration of spermatozoa occurred in the heavier nonnucleated fragments but not even a sperm aster developed. These observations also point to the importance of a discharge of nuclear material into the cytoplasm if cytoplasmic maturity is to be attained.

In exceptional cases like the annelid *Saccocirrus*, the spermatozoon enters the young, by no means full-grown, oocyte and remains passive during its whole growth period (36). It is, however, only after the second meiotic division that the chromosomes of the male nucleus begin to appear as visible units.

In sea urchins the spermatozoa are able to penetrate into the primary oocytes. There is no barrier to polyspermy and no fertilization ensues (106). The oocyte responds, however, through the formation of many hyaline protrusions on its surface. These may be blisters or flamelike, in the latter case recalling the so-called reception cone that is formed after the penetration of a spermatozoon into a ripe egg. (For literature see 245.) The protrusions appearing in the inseminated oocyte withdraw by degrees so that the oocytes may regain their normal appearance.

In the meiotic division stage the eggs are still unprotected against

polyspermy (29). The spermatozoa induce spindle formation but no development ensues. Observations on the eggs of the sea urchin *Brissopsis* indicate that the mechanism which prevents polyspermy is gradually built up. Runnström and Monné (245) observed that polyspermy occurred even in eggs which had completed the meiotic divisions but the reception cones apparently repelled each other. In certain cases only two reception cones were observed; these were situated on opposite poles of the egg (285a,303). This must mean that a change spreads from the site of sperm entry which prevents sperm penetration. In the cases in question, however, the change does not travel rapidly enough to prevent the entrance of an extra spermatozoon at the opposite pole.

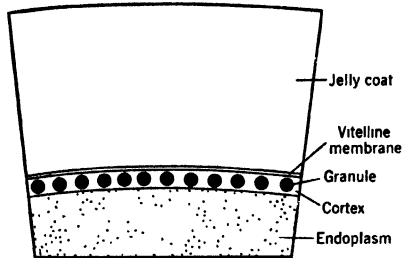


Fig 1. The different layers in the *mature unfertilized* sea urchin egg.

In the sea urchin egg an optimum period of fertilizability is found. In this period a smooth fertilization membrane is rapidly elevated and development is normal. In the stage preceding full maturation the eggs may be called "underripe," while the eggs which have passed the optimum stage are "overripe." In starfish eggs this cycle falls during the stage of the primary oocyte and the period of polar body formation. In sea urchin eggs the cycle is displayed in eggs which have already expelled their polar bodies.

At spawning the eggs are probably in their optimum stage of maturation. The eggs laid by starfish females are in the stage of the breakdown of the germinal vesicle (69,113,235), whereas the eggs laid by sea urchins have completed their nuclear maturation and lost their polar bodies.

Figure 1 will give a preliminary orientation about the different



layers in the sea urchin egg, to which reference will be made often in the following. The jelly coat, which gradually dissolves in sea water, gives rise to "egg water" or "egg secretion." This contains or is identical with "fertilizin," the specific sperm-agglutinating and sperm-activating factor. Artificially dissolved "jelly coat substance" acts also as fertilizin. The vitelline membrane forms part of the fertilization membrane. The cortical granules upon fertilization are incorporated with the fertilization membrane.

Systematic observations and experiments on the cytoplasmic maturation of the sea urchin egg have been carried out by Goldforb (72-74,76-78), Goldforb *et al.* (79,80), and by Paspaleff (211). (See also monograph by Just, 123.) For example, it was shown (77) by the centrifuge method that a viscosity increase occurs in *Arbacia* eggs aging in sea water. The time required to produce a certain stratification of the endoplasm increased in the egg of *A. punctulata* from two to three times the initial value. After 35-40 hours there was a decline in viscosity which preceded death. The latter in its turn entailed a very marked increase in viscosity (77). The increase in viscosity was still higher in the *Paracentrotus* eggs studied by Paspaleff (211). It does not appear from the published data whether a decrease in viscosity also occurred in the *Paracentrotus* eggs. Moreover, it was shown that water permeability increased 30-40% with age (78). A progressive decrease in the resistance to hypotonicity of the aging cells of *Arbacia* was also demonstrated (80).

The writer had ample opportunity on the Swedish west coast to study different degrees of cytoplasmic maturation in sea urchin eggs. Specimens of *Psammechinus miliaris* brought from their natural habitat were kept in wire mesh cages a few meters below the surface of the sea in front of the laboratory. Particularly at the beginning of the season it was easy to follow how the eggs of the different females progressed from an underripe through a ripe to an overripe stage, the rate of formation of the membrane, its optical properties (see Sect. V), and the width of the perivitelline space constituting the criteria of the degree of maturation. At the end of the season freshly caught females with overripe eggs were frequent, but females with underripe eggs were also found (241).

In numerous cases cytoplasmic maturation was followed in eggs removed from the ovaries and stored in normal sea water. Some data from an experiment of this type are given in Table I (237).

TABLE I  
FERTILIZATION OF EGGS OF *Psammechinus miliaris* STORED IN SEA WATER  
AT 22–23°C.

Time after removal from ovary	Reaction on fertilization
(a) 15 min. ....	Strong contraction of egg surface, smoothing after only 10–15 min. Membrane irregularly elevated in form of local blisters for some min.
(b) 1 hr., 45 min. ....	Strong contraction for some min. Smooth, normal, or slightly folded, well-elevated membrane.
(c) 6 hr., 25 min. ....	Slight, rapidly passing contraction. Well-elevated, perfectly smooth membrane.
(d) 10 hr., 10 min. ....	No membranes formed. 15% of eggs fertilized. Expulsions of cortical globules from latter. Vitelline membrane absent.

Normal segmentation occurred in sample *c*, whereas the segmentation in sample *b* was less regular and in *a* and *d* defective and irregular. A strong contraction upon fixation of the spermatozoa is sign of underripeness. In overripe eggs the vitelline membrane (see Fig. 1) gradually dissolves. This was demonstrated by putting the eggs in greatly diluted sea water. Normal eggs burst at one point. The greater part of the endoplasm flowed out forming a projecting plug, an "exovate." The vitelline membrane then became visible outside the contracting cortex (251). In the overripe eggs, on the other hand, no vitelline membrane appeared under similar conditions. The absence of the vitelline membrane is also proved by the direct expulsion of cortical material on fertilization (see Sect. V).

The underripe eggs of *Psammechinus* are anisodiametric (see 76, regarding conditions in *Arbacia*). Both the animal and the vegetal poles of the *Psammechinus* eggs may be marked by light protrusions. These are not present in the fully mature egg, which is also less anisodiametric. The overripe eggs are spherical. Kopac (125) has introduced an effective method of testing the state of vitelline membrane. If it is defective or absent, drops of certain oils may be brought to coalesce with the egg surface.

Behavior of the eggs in a hypertonic solution (2 ml. sea water + 0.6 ml. 2.5 *N* sodium chloride) also varies according to the degree of cytoplasmic maturation (245–247). Eggs in the stage of meiotic divisions present a smooth surface in the hypertonic medium.

In eggs in which nuclear maturation has already taken place behavior varies according to the stage of cytoplasmic maturation. In general when the eggs have shrunk in the hypertonic medium their surface presents numerous fine wrinkles. The wrinkles disappear by degrees, but the time required to attain the smooth state varies according to the egg material. The process which causes the smoothing of the surface in the egg has a certain induction time. Once the wrinkles begin to disappear the smooth state is rapidly assumed (see 246, Fig. 1). The wrinkled eggs are more or less anisodiametric, but simultaneously with smoothing they grow spherical. There is a correlation between the rate of disappearance of the wrinkles and the stage of cytoplasmic maturation. In the underripe eggs the rate is lower than in the mature egg. The subsiding of the wrinkles in different egg materials could well be represented by curves similar to those in Figure 2.

Exposure of eggs in the overripe stage to hypertonic solution gives no wrinkling, but the eggs shrink with a smooth surface. Eggs which behave thus in the hypertonicity test sometimes form upon fertilization a beautiful membrane at the optimum rate, but in general the membrane is low and defective or altogether absent.

The optimum of fertilizability in the sea urchin egg seems to be reached when the viscosity of the endoplasm has undergone a certain increase as compared to the initial state. On the other hand, the egg surface must be liable to a rather rapid liquefaction. The pronounced anisodiametry of the underripe egg mentioned above must depend on a rather high rigidity in the outermost layer of the egg. The gradual change in the shape of the egg into a sphere observed in different species (76) probably also indicates liquefaction of the surface layer. This phenomenon is, in the writer's experience, especially pronounced in the eggs of *Echinocardium cordatum*. The spherical state precedes cytolysis.

It is of interest that absence of calcium and to a lesser degree of magnesium considerably delays the smoothing of the egg surface in the hypertonicity test (246). This may be correlated with the observation of Lindahl (149) and Schechter (254) that the fertilizability of the sea urchin egg is prolonged by exposure to calcium-free sea water.

Wrinkling of the egg probably indicates that the outermost layer of the cortex is in a semisolid state, whereas the smooth stage indi-

cates that the same layer is in a liquefied state. It may be that the changes occur principally in the vitelline membrane covering the unfertilized egg.

The evidence points to the conclusion that the maturation changes in the cytoplasm result from the gradual accumulation of a substance in the egg surface. A certain concentration of this substance might give optimum fertilizability, but a greater concentration entails a decrease in fertilizability encountered in the overripe egg. The same substance may be responsible for the dissolution of the vitelline membrane and the final cytolysis. A certain indication of the action of a substance is given by the following observation: eggs which have assumed a smooth surface in hypertonic solution are not distributed at random among the still wrinkled eggs but are often found collected in groups. The formation of the substance seems to be favored by the presence of the calcium. The results obtained by Delage (55) and Costello (50) indicate that the substance in question is elaborated in the germinal vesicle but is released upon dissolution of the latter. At any rate a nuclear substance is a precursor or the inciting agent in the formation of the substance acting at the egg surface. The difference between starfish and sea urchin eggs with respect to fertilizability may thus be referred to a difference in the rate of diffusion of some nuclear substance.

Whitaker (297) and Tyler *et al.* (278,281) have studied the influence of different additions, such as alcohol, acid, and the like, on the fertilizable period of the eggs of *Urechis* and *Arbacia*. Contamination by bacteria was found to be an important factor in the life span of the unfertilized egg. The aging phenomena in the eggs of *Mactra* have been carefully studied by Schechter (255). Both low and high calcium concentrations are more favorable to longevity than the concentration present in normal sea water. Disintegrating cells give off a toxic substance which accelerates the cytolysis of still intact cells.

Costello (48) centrifuged female sex cells of *Asterias forbesii* suspended on the border between a layer of sea water and isotonic sucrose solution (see 94). It was found that a period of more marked tendency to fragmentation and of decreased viscosity corresponded with the period of maximum fertilizability.

The resistance to normal fertilizability may sometimes be re-

moved by artificial means. The first data along these lines seem to be those reported by R. S. Lillie (143). At the end of the season in Woods Hole he found overripe starfish eggs the reactions of which showed a special inertness. After exposure for three hours to 0.55 *N* sodium chloride solution they were still fertilizable, whereas normal eggs underwent cytolysis after two hours in this solution. Starfish eggs undergo cytolysis after twelve to fifteen hours in sea water. The inert eggs retained their normal appearance in sea water even after 24–48 hours. A large proportion of the inert eggs did not undergo maturation and of these rather few developed to a free swimming stage on fertilization. The inertness could be removed by exposing the eggs to 0.3 volume per cent ether in sea water for one hour and ten minutes. The eggs then showed the normal reaction, including a high percentage of fertilization upon addition of sperm and ensuing development up to the larval stages.

Paspaleff (211) succeeded in rendering overripe *Paracentrotus* eggs with increased cytoplasmic viscosity fertilizable through the addition of 0.5 to 1.0% ether followed by return to normal sea water. No normal membrane formation occurred, however. Ether treatment decreases the viscosity of the cytoplasm. Comparison of sections showed how the finely dispersed ground cytoplasm of the overripe eggs became coarser and vacuolized after exposure to ether. This observed correlation between the fineness of the cytoplasmic structure and viscosity is of great interest.

The refractiveness of underripe eggs of *Psammechinus miliaris* and *Echinocardium cordatum* could be removed by exposure to diluted rabbit or sheep serum (one part serum + nine to fifteen parts sea water) (see 247,249). Changes from 0–100% normal membrane formation were observed on exposure to the diluted serum. The serum caused considerable swelling of the jelly layer. The same was found to hold true for solutions of different amino acids, for example, 0.1% glycine in sea water. Cysteine, tryptophan, glutamic acid, and histidine were also found to be active. Solutions of 0.25 to 0.5% albumin (Fraction V or crystalline albumin of Armour & Co were used) in sea water were found to cause a considerable improvement in fertilization. It has been observed by several authors (see 211) that the jelly layer is thinner and tougher in underripe eggs than in those which present full cytoplasmic maturity. All the agents mentioned have in common that they cause

a considerable swelling of the jelly layer. This may facilitate the penetration of spermatozoa and favor the elevation of the fertilization membrane. But this is not the whole explanation of their improving effect. It was repeatedly found that the fertilization of eggs deprived of their jellies was strikingly improved by treatment with diluted serum or 0.25 to 0.5% serum albumin. The naked eggs of *Strongylocentrotus droebachiensis* tended in general to resist fertilization but in the presence of albumin they exhibited normal membrane formation.

Strong and prolonged contraction often occurred in the inseminated eggs of *Strongylocentrotus*. If the eggs were fertilized in the presence of diluted rabbit serum, 0.25% serum albumin, or 0.1% glycine the contraction was rather slight and of brief duration. Advantage of this experience was taken in work carried out with *Strongylocentrotus* eggs and larvae. The eggs were immersed in a 0.1% solution of glycine in sea water; the pH was regulated to 8.0. The eggs showed normal fertilization. Some minutes after membrane formation they were transferred to normal sea water. In this way normal development was obtained. The untreated eggs exhibited very poor development (300a).

Aging the eggs of *Arbacia lixula* in sea water for several hours often brings about a change in their response to insemination. A thin gelatinous rim appears on the surface of the egg. This represents a step in the process of membrane formation, but the process is not completed. No elevation of the membrane takes place. After further aging the eggs become unfertilizable. If the eggs are kept in contact with 0.25% to 0.5% crystalline serum albumin (Armour & Co.) normal membrane formation occurs even after the control eggs have completely lost their capacity to be fertilized. It is of interest that the addition of 0.0002 to 0.001 *M* adenosine triphosphate (ATP) has the same effect as albumin (300). Even if the eggs already have lost their capacity of forming a normal membrane the addition of albumin or ATP revives this capacity (300). Runnström (236) found earlier that treatment of *Psammechinus* eggs with 0.005 *M* ATP impedes the formation of a normal fertilization membrane. This becomes low and of irregular shape. This was due to the high concentration of ATP used. In the later experiments carried out by Wicklund (300) it was often found that higher concentrations of ATP (0.002 *M*) were less effective in improving the

membrane formation than the lower concentrations mentioned above. It may be pointed out that the concentrations of ATP were not well defined in these experiments because of a partial breakdown of the substance. The concentrations have in fact been lower than indicated.

Nuclear activation is sometimes found in overripe sea urchin eggs. The problem of ovarian parthenogenesis in mammals has been thoroughly discussed by Pincus (216).

Although very specialized cells, spermatozoa, according to Goldforb (75), undergo a maturation which renders them more agglutinable by "egg water," i.e., sea water which has been in contact with eggs. Vasseur (285) stated that the agglutination of *Strongylocentrotus* sperm under the influence of egg water differs during different periods in the breeding season. In the beginning of the season they barely agglutinate. As the season progresses they agglutinate more strongly under the influence of the egg water.

Much attention has been paid to the relation between the fertilizing power of the spermatozoa and the conditions under which they are kept. F. R. Lillie (138;139, p. 134) has carefully examined the question of the relation between dilution and the fertilizing capacity of the spermatozoa of *Arbacia*. Using a fresh sperm suspension he obtained fertilization at dilutions which were fully inactive when the sperm suspension was about twenty minutes old. Lillie prepared a 1% suspension of undiluted sperm from the testicles. This suspension was diluted in a geometric series in powers of two. The dilutions were expressed as  $1/2$ ,  $(1/2)^2$ ,  $(1/2)^3$ , etc. A decline in fertilizability was observed at the power 14–15 in the fresh sperm, whereas in the twenty minute old suspension a decline in fertilizability was already observed at the power 7–8. Lillie maintains that the decline in fertilizability is due to the loss of the agglutinable substance present on the surface of the spermatozoa. According to Lillie, the decline may occur without any concurrent loss of motility. A decline in vitality of the spermatozoa may, however, be difficult to discover merely by observing the movements. The writer observed, for example, that the tail of a spermatozoon moving through the jelly layer or boring at the surface of the egg forms a rather short and condensed spiral very different from the comparatively straight tail of the freely moving sperma-

tozoon. The former manner of movement perhaps requires a higher degree of vitality than the latter.

Cohn (43) arrived at the conclusion that any condition which will paralyze the spermatozoa of sea urchin without killing them prolongs the period of fertilizing power. According to Gemmil (68) the activity of the spermatozoa declines because of the consumption of nutrient substrate. He showed that the length of life of spermatozoa could be increased by adding beef broth to sea water.

Gray (82,83), Carter (37,38) and Hayashi (100a,b) have studied the effect of different factors on the activity of the spermatozoa. The activity was assayed mainly by measurements of the oxygen consumption. According to Gray (83) the decline of activity of a dilute suspension of spermatozoa is due to a process of spontaneous decay or to exhaustion of food reserves. The results obtained may, however, also be interpreted as being due to autointoxication (83). Carter (37) showed that addition of thyroxine prolongs the period of activity of *Echinus* spermatozoa. According to Hayashi (100a) the seminal fluid contains a factor prolonging the period of activity of the spermatozoa of *Arbacia punctulata*. Gray (85) developed a general theory which only assumes the presence of intracellular units of limited but variable viability. Figure 2 shows a number of different curves which Gray (85) constructed on the basis of his assumptions.

E. Wicklund (300) has studied the fertilizing power of sperm of *A. lixula* and of *Psammechinus miliaris*. A suspension containing about 3.5 million spermatozoa was used as a standard. This suspension was diluted to different degrees. The decline of fertilizing capacity could be expressed by curves similar to those represented in Figure 2. Dilution of the suspension changed the form of the curves so that they resembled those at the left of the figure, i.e., the initial constant state was abbreviated or suppressed. If on the other hand 0.05 to 0.5% glucose or fructose was added to a certain suspension the initial state, with 100% or almost 100% fertilization, was prolonged. In one experiment for example with 0.35 million spermatozoa per milliliter the percentages of fertilization were the following, after thirty minutes: (1) control in sea water, 30; (2) sea water with 0.5% glucose, 87; (3) sea water with 0.5% fructose, 100.



Crystalline albumin, 0.2 to 0.4% in sea water, was particularly potent in prolonging the fertilizing life of the sperm. In one experiment with 3.5 million spermatozoa per milliliter fertilization decreased to 50% in 1.5 to 2.0 hours. In the presence of 0.2% crystalline serum albumin fertilization was reduced to 50% only after 12–13 hours. It seems difficult to avoid the conclusion that the albumin serves here also as a nutrient substance. ATP (0.0002 to 0.001 *M*) did not prolong the fertilizing capacity of the spermatozoa, but promoted their motility. The membranes formed in the presence

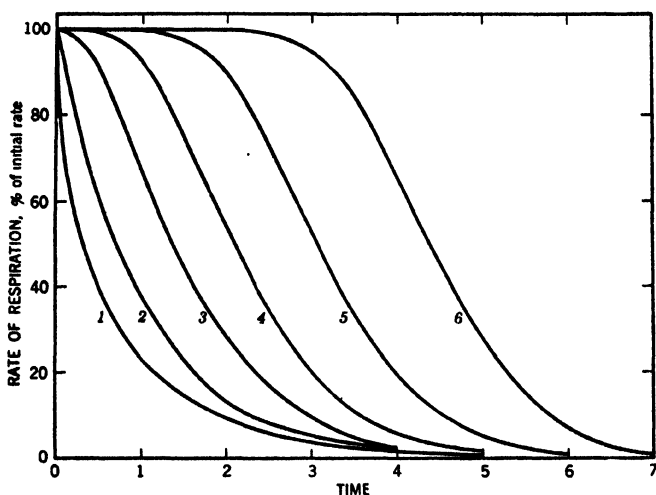


Fig. 2. Curves showing different types of decline of respiration and fertilizing power of sea urchin spermatozoa (modified from Gray, 85). The time is given in hours.

of ATP were also more elevated above the egg surface; they were thicker and smoother in presence of ATP than in ordinary sea water. ATP in this experiment probably acts also on the egg surface (see above) but probably by increasing the vitality of the sperm as well.

With fresh eggs and fresh sperm suspensions of *Arbacia lixula* the membranes were in general well separated from the egg surface. With fresh eggs and aging sperm (3.5 million per milliliter) 100% fertilization could be obtained after 1 to 1.5 hours, but the fertiliza-

tion membranes were not separated from the egg surface. The membrane was present only as a gelatinous layer on the surface of the egg (see above). This result demonstrates that a gradual decrease in the vitality of the spermatozoa occurs. In a certain stage of aging, vitality is still sufficient to induce 100% development but not to incite normal membrane formation. The eggs with incomplete membrane formation developed into larvae which were only slightly less normal than those of the control.

Very important work on the physiology of the spermatozoa of fowl, mammals, and man has been carried out in recent years. In these organisms the seminal fluid plays a great role in the nutrition of the spermatozoa. In a number of mammals examined, including man, Mann (174) found the main nutrient substrate to be fructose, which is secreted by the male accessory glands. The survival may be maintained both by fermentation and oxidation of fructose. According to Chang (41), who worked with rabbits, the fertilizing power is better preserved in a concentrated than in a dilute suspension of spermatozoa. The rather rapidly enlarging field of the physiology of spermatozoa in higher vertebrates has recently been reviewed by a number of specialists in this field (56). It is therefore not necessary to enter into details, particularly since the results so far do not contribute essentially to the problems with which we are primarily concerned in this article.

### III. Interacting Substances Produced by the Germ Cells

Study of the problem of interaction between substances produced by the germ cells was initiated mainly by F. R. Lillie, working with *Nereis limbata* and sea urchins, particularly *Arbacia punctulata*. The literature preceding his work is reviewed in his book (139), in which a clear presentation of the author's own results and conclusions are also given (see also Tyler's extensive review, 277). Only the main points of Lillie's views will be given here. The presence of a substance secreted from the eggs can be demonstrated in the sea water surrounding them, the "egg water" (see Sect. II). This substance may be assayed by its agglutinating effect on the spermatozoa of its own species. The agglutinating substance is identified with "fertilizin," one of the main components in the "fertilization reaction." The stimulating effect of egg water on the movements of the spermatozoa is also ascribed to fertilizin. The possibility of

dissociating the stimulating and the agglutinating effect is, however, recognized (139, p. 112).

The fertilization reaction proceeds in two stages. After having reacted with a receptor present in the surface of the spermatozoon, fertilizin becomes capable of reacting with an egg receptor. When the compound sperm receptor-fertilizin-egg receptor has been formed, the egg is activated. The union sperm receptor-fertilizin is revealed by the agglutination of the spermatozoa and by the fact that the agglutinating substance—the fertilizin—can be removed from a solution by shaking it with a sperm. The second part of the reaction, linking of the sperm receptor-fertilizin complex with the egg receptor, is demonstrated according to Lillie by the block to fertilization produced by blood of the same species. This block does not disturb the agglutination of the spermatozoa and thus fails also to affect the reaction sperm receptor-fertilizin (139). A block similar to that produced by blood is likewise produced by copper in very low concentrations (140a). Moreover, Lillie demonstrated the presence in the interior of the egg of a substance capable of blocking the reaction between sperm receptor and fertilizin. This substance was designated "antifertilizin." Lillie proposed the additional hypothesis that fertilization brings about the union of antifertilizin with the fertilizin which has not reacted with the sperm receptor. Lillie's theory combines a number of important results in a consistent scheme. These results demonstrate to us certain specific reactions which may play a part in the specificity which prevails in fertilization. On the other hand, certain points remain hypothetical and the connection between the formation of the ternary complex sperm receptor-fertilizin-egg receptor and the physiological processes in the fertilized egg remains obscure.

In the meantime experience had been gained concerning the formation of substances acting in the fertilization of algae. These results will not be described in detail here. They have recently been reviewed by a pioneer in the field, Hartmann (88), and also by Sonneborn (258). The last-named author also takes up for consideration interesting recent work on sexual substances in protozoa, but this also falls outside the scope of this article.

A great wealth of interesting results was obtained by Moewus, Moewus, and Kuhn and their co-workers concerning the unicellular alga *Chlamydomonas*. It was demonstrated that the filtrate of

illuminated cells contains a substance which renders the gametes mobile even in the dark or under anaerobic conditions. Besides this motility-stimulating substance, the filtrate also contains male and female substances which cause aggregation of the cells and probably also favor the fusion of male and female gametes. In a brilliant piece of research Kuhn, Moewus, and Jerchel (130) have elucidated the chemical nature of the agents. Interacting substances secreted by the gametes have been called "gamones" by Hartmann and Kuhn (see 91). The female substances are the "gynogamones" and the male substances the "androgamones." Hartmann considers that gamones are probably generally involved in the fertilization process. In sea urchins Hartmann and co-workers (91,92) recognized the following gamones and gamone effects:

Gynogamone I stimulates the movements of the spermatozoa and attracts them chemotactically.

Androgamone I lames the movements of the spermatozoa.

Gynogamone II agglutinates the spermatozoa.

Androgamone II dissolves the jelly coat of the eggs and inhibits the agglutinating activity of gynogamone II.

Gynogamone I and androgamone I are antagonists with respect to their action and the same holds true for the pair gynogamone II and androgamone II. Both pairs of antagonizing gamones have been demonstrated by Hartmann and others in a number of different organisms (90,176,177,253).

It is obvious that gynogamone II corresponds to Lillie's fertilizin and gynogamone I to the activating substance which Lillie also recognized as possibly different from the agglutinating substance (see also 164). It may be pointed out that Hartmann's gamone theory aimed only at explanation of the attraction and copulation of the gametes. Lillie's fertilizin theory, on the other hand, also included the activation of the egg. I shall now attempt to give a more detailed survey of the further development of our knowledge concerning the substances which interact in fertilization (see also 277).

The activating substance in the egg secretion of *Arbacia lixula* which stimulates the sperm motility was identified by Kuhn and Wallenfels (131) in their work with *A. lixula* as echinochrome, a pigment isolated earlier (5), which is present within the egg. Leucoechinochrome was identified by the German investigators as 1,3,4,5,6,7,8-heptaoxy-2-ethylnaphthene. They (132) demonstrated

further that the pigment is bound in the jelly coat to a macromolecular symplex made up of two components. In a recent paper (90) on the interacting substances from the gametes of *Salmo iredeus*, the sperm-activating substance is identified with a carotenoid, astaxanthin. It was already recognized that carotenoids have an activating effect on the gametes of *Chlamydomonas* (see 88,258). It is assumed that in many organisms, *Paracentrotus lividus*, *Pecten maximus*, etc., the sperm-activating substance is identical with the carotenoids present in these eggs.

Objections to the results obtained by the German workers on *A. lixula* have been raised by Cornman (47), who did not find an activating effect of echinochrome on *A. punctulata* sperm. Cornman dialyzed the sperm-agglutinating egg water and arrived at the conclusion that two sperm-activating substances may be present, one dialyzable and one which is more closely attached to the agglutinating substance. Vasseur and Hagström (286) conducted experiments on egg water of *Echinocardium* with similar results. Both the dialyzing factor and that remaining within the cellophane bag activate the sperm. The latter also agglutinates. It seems probable from these observations that the high molecular component may also exert a stimulating action. The dialyzable factor may perhaps simply be a breakdown product of the former. It has already been described above how different additions may activate sperm (for further discussion see Sect. IV). Glaser (71) contended that a lipase is present in the egg water from *Arbacia*, but this writer's efforts to demonstrate a lipase in egg water of *A. lixula* were negative.

The sperm-laming substance of Southwick (259) and Hartmann and Schartau (91) can be extracted from the sperm by methanol. This factor is low molecular and thermostable (see also 90,243,251). Its biological importance seems to be doubtful in some cases; in others, such as the salmon, it may play a role.

The sperm-agglutinating substance, fertilizin, has been the subject of a number of papers. With regard to its source, Lillie considers that it is secreted from the egg and stored in the jelly layer surrounding it. Lillie maintained that fertilizin is secreted from the eggs of *Arbacia punctulata* (139) and *Strongylocentrotus franciscanus* (140) even if the jelly layer is removed from the egg. This opinion is, however, not supported by subsequent work and can scarcely be regarded as tenable, even though further examination

of the question is desirable. Tyler (271) showed that in *S. purpuratus* and *Megathura crenulata* the sperm-agglutinating substance is located in the jelly layer surrounding the egg. (The reader is referred to the more detailed discussion in Tyler's review (277, p. 190). When this layer was removed by means of acid sea water (pH 3.5 to 4.5) or a 1% chymotrypsin solution in sea water, no agglutinating action was obtained from the jellyless eggs even after prolonged contact with sea water. Hartmann, Schartau and Wallenfels (92) report that fertilizin (gynagamone II) is given off from the eggs of *A. lixula* only as long as the jelly layer or remnants thereof are present around the eggs. Working with *S. droebachiensis*, *Paracentrotus lividus*, and *Echinus esculentus*, Vasseur (285) has confirmed that eggs with the jelly layer completely removed do not secrete any fertilizin. It was possible to find specimens of *E. esculentus* which contained only oocytes. The jelly layer of these oocytes was brought into solution by exposure to acid sea water. It was shown that the jelly from the oocytes also exerted fertilizin activity. This is contrary to another of Lillie's contentions, namely, that production of fertilizin starts only after the maturation of the egg.

The mode of formation of the jelly coat has likewise been a subject of controversy. According to some authors, it is secreted from the follicle cells, while others maintain that it is a secretion product of the growing oocyte. A great number of fine pseudopodia emanate from the vitelline membrane of the growing oocyte and penetrate the increasing jelly layer. (For literature and discussion see 148,245.)

Wallenfels (287) was the first to suggest, but without publishing the experimental evidence, that the jelly coat is rich in mucin. According to Tyler and Fox (279) the jelly layer is "either protein or very closely associated with protein." Both in the snail *Megathura crenulata* and in the sea urchin *Strongylocentrotus purpuratus* the nondialyzable fertilizin is precipitated by the addition of ammonium sulfate without losing its activity. Active concentrations give the common color reactions for proteins. Runnström, Tiselius, and Vasseur (252) definitely demonstrated the presence of a carbohydrate component.

As in the previous work (132,279,287), the jelly coat substance was obtained in solution by treatment with acid sea water and

then precipitated with ammonium sulfate and dialyzed. Ultracentrifuging of a solution containing 0.264 mg. nitrogen per milliliter of 0.2 *N* sodium chloride gave a well-defined main component with sedimentation constant  $2.90 \times 10^{-13}$ . Besides this a small amount of a more rapidly sedimenting substance was present. The sedimentation constant is, however, dependent on the concentration. This is characteristic of elongated gel-forming molecules. Estimation of the molecular weight is therefore difficult. The jelly coat substance exhibits a strong birefringence of flow, but because of cross-linking of the molecules this method also failed to give definite data concerning the molecular weight.

In the electrophoresis apparatus the jelly coat substance rapidly migrated as a single component toward the anode. The electrophoretic data are summarized in Table II. From the data available it

TABLE II  
ELECTROPHORETIC MOBILITY AT 0.5° OF DISSOLVED JELLY COAT SUBSTANCE  
OF *Echinocardium cordatum*

Buffer*	pH	Mobility
Acetate	3.76	$-13.2 \times 10^{-5}$
Acetate	4.67	$-14.2 \times 10^{-5}$
Phosphate	6.02	$-16.2 \times 10^{-5}$

\*Ionic strength 0.1.

seemed probable that the jelly coat was composed of a glycoprotein with an acid carbohydrate compound. This has been more fully shown by Vasseur in work on the species *Strongylocentrotus droebachiensis*, *Paracentrotus lividus*, *Echinocardium cordatum*, and *Echinus esculentus* (282,284) and by Tyler in work on *S. purpuratus*.

Vasseur precipitated the dissolved gelatinous substance either with ammonium sulfate or with alcohol and ether. The fibrous precipitate is easily collected with a glass rod or a spoon. This precipitate is dialyzed in distilled water until completely free from chloride and sulfate ions. During this process the substance dissolves and a very viscous solution is obtained. The ammonium sulfate precipitate dissolves more readily than the alcohol and ether precipitate. The jelly coat substance is obtained in a very suitable dry form by lyophilizing this concentrated solution.

The chemical nature of the carbohydrate in the jelly coat was investigated with the orcinol and sulfuric acid reaction on carbo-

hydrates (see 283). The color curves obtained in the three first-named species conformed with the curves for methylpentose. Quantitative determinations with chromic oxide oxidation confirmed the conclusion that the carbohydrate component was polymethylpentose and gave about 45% of this compound. Recent work by Vasseur and Immers (286a) on the chemical composition of the jelly coat substance for different sea urchin species has demonstrated a genus specificity of the carbohydrate component, inasmuch as the polysaccharide was shown to be composed solely of galactose residues in *Echinus esculentus* and of fucose in *Echinocardium cordatum*, but of fucose and galactose in *Strongylocentrotus droebachiensis* and of fucose and glucose in *Paracentrotus lividus*, fucose being the dominating monosaccharide in the jellies of these latter species.

A content of 8–11% sulfur corresponding to 24–33% sulfate was obtained (284). Most of the sulfur was retained in the ash as sulfate. Ash content was 20–50%. The total Kjeldahl nitrogen was 2–5%, depending on species and preparation. The figures for carbohydrate, sulfate, ash, and protein accord well with a protein-carbohydrate complex consisting of 25% protein and 75% polymethylpentose or polyhexose esterified with one sulfate group per monosaccharide residue. Tyler and Fox (279) have given somewhat higher values for the nitrogen content of the jelly substance from *Megathura* and *Strongylocentrotus*, namely, 4.5 and 5.2%, respectively. An even distribution of the sulfuric acid groups over the whole polysaccharide component is borne out by further work on jelly solution of *Echinus esculentus* (Vasseur, 285). Only very few configurations of the polysaccharide part seem to be possible on the rather reasonable assumption of a pyranose structure of the monosaccharide residues, e.g., a straight chain of galactose residues connected with 1,6' linkages and the sulfuric acid group in position 3. In the fucose containing jellies the 1,6' linkage between the fucose residues naturally is not possible. In *Strongylocentrotus droebachiensis* and in *Paracentrotus lividus* a branched chain structure seems very likely, with a main chain of galactose and glucose residues, respectively, connected with 1,6' linkages, and with branches composed of fucose residues.

Several amino acids have been estimated by microbiological methods in the acid hydrolyzate. Valine seems to dominate. According to Wallenfels (287) a base rich in nitrogen can be separated from the jelly coat substance of *Arbacia lixula*. Vasseur's data,



however, provide no evidence that the protein component would have a pronounced basic character. According to Tyler (277,277a) hexosamine is present to an extent of at least 5% and galactose has been identified as the osazone in the jelly substance of *Strongylocentrotus purpuratus*.

Vasseur and Immers (286b) are investigating the question of the presence of hexosamine in the jelly substance more closely at present. They have found that the jelly hydrolyzate gives the typical red color with *p*-dimethylaminobenzaldehyde after heating in a sodium carbonate solution alone, the addition of acetylacetone not being necessary. An identical color is obtained when lysine or ornithine are heated in a sodium carbonate solution together with an excess of carbohydrate, *e.g.*, glucose and fucose. This indicates that the positive color reaction for hexosamine obtained with jelly hydrolyzate (*cf.* 277,277a,284) in reality depends on an interaction of lysine, etc. with carbohydrates rather than on the presence of hexosamine.

The sensitivity of the sperm-agglutinating action of fertilizin to trypsin and chymotrypsin (279) points to the presence of an active protein component. Vasseur (285) did not find any inactivation by trypsin of the fertilizin of *Paracentrotus*. This does not disprove the preceding conclusion, since the protein in the jelly coat substance of *Paracentrotus* may be protected by certain linkages, or it may exert an inhibiting action on the enzyme (see Sect. VIII). Vasseur (see 121) stated, however, that periodate inhibits the agglutinating action of *Echinus* fertilizin. This inhibition has to be due to partial destruction of the carbohydrate component. Consequently there are some indications that both the protein and the carbohydrate components are essential to fertilizin action, but wider study of this problem is necessary. Some analogies with the specific substances of the blood groups may be pointed out here. Amino acids have been found to be components of the blood group factors and these seem to play a role in determining the serological specificity (260).

It is also interesting to note that fucose, a component of blood group substances, was found in the jelly coat of several sea urchin species.

Vasseur (284) found the amino acid composition of *Echinocardium cordatum* jelly differed considerably from that of the three regular

sea urchin species examined (*Paracentrotus lividus*, *Strongylocentrotus droebachiensis* and *Echinus esculentus*). These results may reflect biochemical differences between representatives of different suborders (irregular and regular sea urchins).

According to Kuhn and Wallenfels (132) only a part of the jelly coat substance, the "Hilfsträger," is identical with the agglutinin (gynogamone II). The evidence points rather to the view that the jelly coat substance is uniform in the species examined by Vasseur.

Immers and Vasseur (121) found that dissolved jelly coat exerts a considerable inhibitory action on the blood-clotting process (see also 120b). Its activity is about  $1/20$  of that of heparin. The observations in question were suggested by the similarity in chemical structure of jelly coat substance and heparin, particularly with respect to the high sulfate content of both.

Vasseur (284a) found in *Strongylocentrotus droebachiensis* that the fertilizin of normal egg water is more potent in causing cluster formation in a sperm suspension than the fertilizin obtained by acid dissolution of the jelly coat. This depends, in his opinion, on the presence of inactive polysaccharide molecules in the jelly solution, whereas the normally secreted egg water consists almost entirely of active protein-polysaccharide complexes.

In *S. droebachiensis* the limiting concentration of fertilizin giving agglutination is of the order of one part per million (Vasseur, 284a), which is of about the same magnitude as given by Wallenfels (287) for *Arbacia lixula* and by Tyler (277) for *S. purpuratus*.

Hyaluronidase from the testicle of the bull does not break down the jelly coat (247). Lundblad and Monroy (168), following the method used by Madinaveita (173) for the preparation of mucinase from *Crotalus*, extracted the sperm of *Arbacia* and *Paracentrotus*, but this preparation remained without effect on the viscosity of a solution of jelly coat substance. On the other hand, it produced a rather slow decrease in the viscosity of a solution of potassium hyaluronate (see also 223). Lundblad (167) showed further that the Madinaveita preparation contained a gelatin-liquefying enzyme.

Metz (178) showed that agglutination of starfish spermatozoa by fertilizin from starfish occurred only after the addition of egg white as an adjuvant (see also 242). According to Vasseur (284a) the addition of isotonic calcium chloride strongly enhances the agglu-

tion of *Strongylocentrotus droebachiensis* spermatozoa by fertilizin from the same species. In many cases no agglutination occurred at all (see above) without the addition of calcium chloride (for example, 1 part jelly coat solution + 1 part isotonic calcium chloride). A somewhat less pronounced calcium effect was also found in *Echinus esculentus* and *Paracentrotus lividus*.

Southwick (259) and Frank (65) obtained from spermatozoa of *Arbacia punctulata* an extract with the following effects: (1) it neutralized the sperm-agglutinating effect of fertilizin; (2) it precipitated and agglutinated the jelly layer of the egg; and (3) it agglutinated the naked eggs. Frank heated the spermatozoa for three minutes; after centrifuging, the supernatant contained the active principle. Hartmann, Schartau, and Wallenfels (92) showed that the substance (androgamone II) is contained in the methanol-insoluble part of the spermatozoa.

Tyler and O'Melveny (280) acidified (below pH 6) sperm suspensions from *Strongylocentrotus purpuratus*. After centrifuging they found the supernatant active in neutralizing fertilizin. Tyler proposed calling the acting substance "antifertilizin," thus adopting a designation already used by Lillie (139) for an agent present in the egg. According to Tyler and O'Melveny antifertilizin is a protein which is nondialyzable, precipitates with ammonium sulfate, and is inactivated by heat and acidity. Furthermore, it gives the common (xanthoproteic, Millon and biuret) color tests for protein. Runnström, Lindvall, and Tiselius (243) lyophilized the sperm, extracted with methanol, discarded the methanol extract, and extracted the residue according to Frank (65) with sea water at 90° for three minutes. Thereafter the solutions were dialyzed against sea water or buffer solution. Electrophoresis demonstrated a uniform compound migrating toward the anode. The optical properties of the solution indicated the presence of nucleic acid. Through dialysis against acetate at pH 4.2 the extracts could be freed from considerable amounts of thymonucleic acid. The great similarity between the action of the antifertilizin and clupein solutions pointed to basic protein as the active principle. Finally, Hultin (115,116), following classical methods (256), extracted a basic protein fraction from the nuclei of the spermatozoa. He demonstrated that his fraction was identical with antifertilizin. Hultin's work was carried

out on the following species: *Brissopsis lyrifera*, *Echinocardium cordatum*, *Patella vulgata*, and *P. coerulea*.

Hultin used the acid extraction method inaugurated by Tyler and O'Melveny. Inactive material, possibly histones (see 115,116), were precipitated by bringing the extract to pH 10.00. After separation of the nonactive fraction (histones), the antifertilizin-active fraction was precipitated from the dialyzed supernatant by a mixture of picric acid and sodium picrate. The precipitate was dissolved in 80% acetone, alcohol was added, and the active substance was finally precipitated by 20 volume per cent sulfuric acid. Hultin showed, moreover, that extraction of the nucleoprotein complex of the sperm nucleus according to Pollister and Mirsky (218) gave an antifertilizin-active product. On the other hand, an extract from the cytoplasm following the method of Stoneburg (264) as applied to the spermatozoa by Pollister and Mirsky (218) gave no antifertilizin activity. The nuclei remaining after extraction according to Stoneburg were treated with 0.1 N hydrochloric acid. After neutralization the extract showed a strong antifertilizin activity. The extracts obtained both according to Frank and according to Tyler and O'Melveny were shown by Hultin to contain thymonucleic acid. The anodic behavior of the sperm extract observed by Runnström *et al.* (243) was no doubt due to the presence of nucleic acid.

TABLE III  
AMINO ACID COMPOSITION OF ANTIFERTILIZIN FROM SPERM

Amino acid	<i>Patella vulgata</i>		<i>Patella coerulea</i>		<i>Arbacia lixula</i>	
	Amino acid residues, %	No. of residues	Amino acid residues, %	No. of residues	Amino acid residues, %	No. of residues
Arginine	33.6	40	60.1	80	16.0	14
Lysine	22.5	32	9.3	14	27.6	30
Histidine	0.77	1	0.0	0	1.04	1
Alanine	8.5	20	3.5	9	17.3	30
Glycine	5.5	15	4.9	14	10.7	22
Isoleucine	1.2	2	0.56	1	3.5	4
Leucine	3.6	6	3.4	6	4.2	5
Proline	8.6	15	3.3	6	9.2	12
Serine	7.7	15	9.9	21	6.0	9
Threonine	4.0	7	1.9	3	2.2	3
Valine	4.1	7	3.2	6	2.2	3
<i>Min. molec. wt.</i>	20,800		22,000		15,500	

It should be pointed out in this connection that Wallenfels (287) had already carried out certain steps toward the purification of androgamone II (antifertilizin). In these a fractionated precipitation with phosphotungstic acid was involved. It seems fairly clear from the procedure followed by Wallenfels (Dr. Wallenfels has kindly given me his elaborate unpublished data) that the active substance had the character of a base.

Using largely microbiological methods, Hultin and Herne (120) also assayed the amino acid content of antifertilizin from different species. Table III gives the percentage of different amino acid residues in the antifertilizin molecule of some species. Similar assays were made with the sea urchins *Brissopsis lyrifera* and *Echinocardium cordatum*. The results are almost identical with those found in *Arbacia*. It is therefore all the more surprising to find such great differences between the sperm antifertilizins from the two snail species of the genus *Patella*. In these arginine dominates, whereas in sea urchins lysine is the amino acid present in highest concentration.

Tyler has approached the problem of the role of antifertilizin in fertilization by serological methods. Antifertilizin from the sea urchins *Strongylocentrotus purpuratus* and *Lytechinus anamesus* (280) were injected into rabbits in order to produce antisera. Fertilization in the sea urchins was suppressed in the presence of the antisera, whereas normal rabbit sera did not impede it. The sperm concentration was adjusted to a number giving exactly 100% fertilization in the control. Tyler (276) later repeated these interesting experiments with *Lytechinus pictus* and the geophycean worm *Urechis caupo*. To preclude inhibition of fertilization by mere entangling of the spermatozoa, Tyler made use in his more recent work of "univalent" antibodies. Samples of the antisera having agglutinating titers of 512 to 2048 were subjected to photooxidation (274,275). Under appropriate conditions the antisera lose their agglutinating power but retain the capacity to react with the antigen, thus preventing the agglutinating reaction with the nonphotooxidized polyvalent antibodies.

The univalent antibodies also inhibit fertilization. These results no doubt prove that specific high molecular substances are involved in fertilization. The degree of purity of the antifertilizin used even in Tyler's later research was, however, not sufficient to ascertain

beyond question that the antifertilizin, the extractable fertilizin-neutralizing substance, acted as the only antigen in the immunization experiment. The extract certainly contained nucleic acid to which not only antifertilizin but also other proteins (for example, histones) were bound (see also 277).

Hartmann *et al.* (91) did not find the reaction between fertilizin and antifertilizin to be species specific. Runnström and his group had the same experience. Hultin (115) discusses the possibility that besides the nonspecific nuclear antifertilizin there may be a specific sperm receptor present in the surface of the spermatozoon as was assumed by Lillie. In crude extracts the specific reaction might be covered by the nonspecific reaction with nuclear basic protein. Hultin also tried without success to devise a method of distinguishing between the specific and the nonspecific reaction. Thus Lillie's specific sperm receptor has so far not been obtained in extract. On the other hand, its presence in the surface of the spermatozoon cannot be doubted.

Runnström (235) showed that extracts made from sperm of *Asterias rubens* according to Frank's method (65) (see above) did not precipitate the jelly layer of the *Asterias* egg. The extract, however, precipitated the jelly layer of eggs of *Psammechinus miliaris*. Borei (24), while confirming these results for extracts from the sperm of *A. glacialis*, found that the sperm extract in question is able to agglutinate the surface of the naked eggs both of *Psammechinus* and *Asterias*. Thus the naked egg surface of *Asterias* is similar in reaction to that of the sea urchin, although the jelly behaves differently. The purified antifertilizin from sea urchin sperm was also shown to cause agglutination of the tails of the spermatozoa (116). Sperm antifertilizin thus behaves as an "auto-antibody" according to Tyler's concept (277, p. 205). Clupein is not able to cause tail agglutination. If, however, spermatozoa are exposed to clupein combined with nucleic acid, definite tail agglutination occurs. Both clupein and sperm antifertilizin activate the sea urchin eggs (see Sect. VI, below).

The chemical nature of antifertilizin from eggs (139,270a) is still unknown (for further discussion, see 277).

Hartmann, Medem, Kuhn, and Bielig (90) showed that in *Salmo irideus* the sperm-agglutinating factor (gynogamone II, according to the nomenclature of the authors) has the nature of a globulin.

They made the interesting observation that an inhibitor of the fertilizin is present in the egg. This agrees with Lillie's observation of the presence of an antifertilizin in the sea urchin egg.

From *Salmo* sperm a factor has been extracted which counteracts sperm agglutination. The substance in question, androgamone II or sperm antifertilizin, is, like the corresponding substance from the sea urchin, insoluble in methanol and heat stable.

The experiments of Lillie (139) on the inhibitory action of *Arbacia* blood on fertilization in the same species have been critically re-examined by Pequegnat (217). The inhibitor is not normally contained in the blood but certain blood cells are the source of the inhibitor. According to data presented by the author fertilizin is not able to neutralize the inhibitor, a result contrary to Lillie's contentions (see above).

#### IV. Incorporation of the Spermatozoon. Species Specificity in Fertilization

Only brief reference will be made here to the mechanisms by which the spermatozoa penetrate the exterior hulls to arrive at the cytoplasmic surface of the egg. The dispersion of the follicle cells which follows upon fertilization of rabbit ova has been ascribed to enzyme action (see 216, p. 75). McClean and Rowlands (170) have in fact demonstrated that dispersion of the cumulus cells is due to the action of hyaluronidase present in the spermatozoa. (For further references concerning this question see 60,265,266.) The field has been reviewed by Favilli (59).

Detailed descriptions have been given of the mechanism in marine animals which enables the spermatozoa to get through the hulls surrounding the egg. In *Asterias*, filaments attach themselves to the spermatozoon, shorten, and draw the latter inside the egg (39,40, 113). In the egg of the annelid worms *Nereis limbata* (49,202) and *Pomatoceros triqueter* (189) the spermatozoon also penetrates into the egg through a cone or filament which remains attached to the vitelline membrane, while other filaments are withdrawn after attachment of the spermatozoon.

Tyler (270) demonstrated the presence of a lytic agent in the spermatozoa of two snails, *Megathura crenulata* and *Haliotis crach-*

*erodii*. Cross lysis did not occur between the species. According to Monroy (189), lytic action is exerted by the spermatozoa on the vitelline membrane in *Pomatoceros*. Hartmann *et al.* (92) maintained that antifertilizin from *Arbacia* spermatozoa (androgamone II) dissolves the jelly coat of the egg and thus facilitates the entrance of the spermatozoon. Other authors, including Frank (65), Tyler, and O'Melveny (280), and Runnström and collaborators (243,252) have found antifertilizin to have only precipitating action. In Hartmann's material, *Arbacia lixula*, also, sperm antifertilizin precipitates the jelly. In the writer's experience slightly precipitated jelly coats become brittle and may burst, releasing the egg. From the description given by Hartmann *et al.* (92, p. 410) it seems that their conclusions were partly based on similar observations. On the other hand, abundant living spermatozoa of *Arbacia* are able to break down the jelly, as described by these workers.

As reported in Sect. III no "mucnase" attacking the jelly coat was extracted from sea urchin sperm. On the other hand, a gelatin-liquefying action was present in the extract prepared according to Madinaveita. The same enzymic agent appears also after extraction with weak salt solution. This agent may assist in the dissolution of the jelly coats under the attack of numerous living spermatozoa. In systematic experiments Vasseur (285) found that mechanical agitation removed the jelly coats from the fertilized egg much more easily than from the unfertilized egg. This only confirms an experience undoubtedly shared by many workers in the field.

According to Lillie (139) the attachment of the spermatozoa to the egg surface is a more-or-less specific agglutination of the spermatozoon to the egg. In this the same specific reaction between fertilizin and sperm receptor would be involved as in the agglutination of spermatozoa by fertilizin. As already pointed out, Hartmann (see 88) holds the opinion that interaction between the "gamones" is operative in the fusion of the male and female gametes. Elster (55a, page 70) found that the very difficult cross fertilizations with *Arbacia lixula* spermatozoa are generally facilitated by means of adding egg water from *Arbacia lixula*. Hartmann refers to the following experiment (88, p. 392): Crossing of *Paracentrotus* males and *Arbacia* females produces no or very low fertilization. The *Arbacia* eggs were, however, shaken and washed to remove the jelly layer. They were then inseminated in egg water from *Paracen-*



*trotus*. On a second attempt the comparatively high cross fertilization of 20% was obtained.

In the methanol extract of lyophilized sperm of sea urchin, Runnström, Tiselius, and Lindvall (251) found a substance with definite influence on the egg surface. This factor was designated androgamone III (251) or sperm lysin (249). The first designation considered (251, p. 1), "surface liquefying sperm factor," would perhaps have been more suitable, but the one finally selected has the merit of brevity. Unfertilized eggs pretreated with sea water containing sperm lysin present a smooth surface when exposed to hypertonic sea water (2 ml. sea water + 0.6 ml. 2.5 *N* sodium chloride). The control eggs in hypertonic sea water without lysin become finely wrinkled (see Sect. II). Attempts were made at purification of the sea urchin sperm lysin (242), but shortage of material rendered this task difficult. According to experiments carried out by D. Cheesman, the sperm lysin preparations gave surface films in the air-water interphase. On compression a force-area curve resembling those to be expected from a mixture of lecithin (or lysolecithin) and oleic acid was obtained see (242, p. 7). From mature mackerel testicles, which were available in great quantities, an agent was isolated which exerts the same action on the egg as sperm lysin from the sea urchin sperm. The dominating compound of this agent was a fatty acid with eighteen carbon atoms. The uptake of bromine indicates the presence of four double bonds in the molecule (161). The acid is present in the intact cell in a combined form, probably as a component of a phosphatide.

Cephalin extracted from sea urchin sperm had a certain weak effect in the hypertonicity test. The eggs wrinkled in the cephalin solution (*e.g.*, 0.05% in sea water), but smoothing occurred earlier when cephalin was present than in pure hypertonic sea water. Other observations also point to the conclusion that sperm lysin is not identical with cephalin but possibly with some of its breakdown products. Detergents such as Duponol or Dupont QB, act in the same way as sperm lysin, which likewise has the character of a detergent. It penetrates into protein and lipoprotein surface films with expulsion of the protein component. Solid protein films are liquefied in this process (242). This is keeping in with the smooth plasmolysis of the egg surface in the presence of sperm lysin. This type of plasmolysis is due to liquefaction of the egg surface.

Even in strong dilutions sperm lysin as well as the synthetic detergents inhibit fertilization without immobilizing the spermatozoa. These carry out boring movements on the egg surface but do not become attached. Aged but not fresh preparations of cephalin from sea urchin sperm inhibited fertilization. Eggs and sperm of *Psammechinus miliaris* and *Echinocardium cordatum* were used in these experiments. The detergents, including sperm lysin, not only inhibit attachment of the spermatozoa to the egg surface but also prevent agglutination of the spermatozoa by egg water in experiments with *Echinocardium* (242).

These results may be interpreted to favor the idea that a reaction between fertilizin in the egg surface and a sperm receptor causes the attachment of the spermatozoon. The results further suggest that release of a surface-active substance in the egg surface might form part of the mechanism preventing polyspermy. Monroy (187) suggests that the surface-active substances act by splitting lipoproteins, which seems possible in view of the reported action on a lipoprotein film (see also Macheboeuf and Tayeau (171,172), who demonstrated that unsaturated fatty acids are able to split lipoproteins).

The problem of species specificity in fertilization has been approached by studying the results of cross fertilization. This very extensive subject has been most ably reviewed by Lillie (139) and by Lillie and Just (141). The problem of interest in our discussion would be whether there is a close parallelism between the degree of cross fertilization and the interaction between fertilizin and sperm receptor. Even if a general parallelism exists, our knowledge is not detailed enough to decide how close it is.

There is still another phenomenon in which specificity is involved, namely autosterility. The best-known case among animals is the ascidian *Ciona intestinalis*, thoroughly analyzed by Morgan. He found (197) that the eggs can be fertilized by sperm from the same individual if the membrane surrounding the egg is removed. The specific hindrance thus resides in the membrane. Reverberi (220) showed that the membrane likewise constitutes a hindrance to cross fertilization. Reverberi's experiments were carried out with the combination of *Ciona intestinalis* females and *Phallusia mamillata* males. Minganti (180) has considerably amplified Reverberi's work. Combining a number of different ascidians, Minganti never obtained

cross fertilization without removal of the membrane. This held true even in the case of species which are normally autofertilizable.

Hultin (117) removed the vitelline membrane from eggs of *Psammechinus microtuberculatus*, *Sphaerechinus granularis*, and *Paracentrotus lividus*. This was effected by treatment of the eggs with trypsin or ion-free isotonic urea solution. The eggs devoid of their vitelline membranes were then inseminated with sperm from some of the other species mentioned. Even when the "normal" cross fertilization was very low, the eggs devoid of vitelline membrane gave a percentage of fertilization which came close to the result of insemination with sperm of the same species.

Eggs of *Arbacia* were also divested of their vitelline membranes. If inseminated with *Sphaerechinus* spermatozoa these eggs gave 60–90% cross fertilization, although normal *Arbacia* eggs were fertilized by the foreign sperm only in a few cases (118). *Arbacia* eggs without their vitelline membrane gave 30–90% fertilization when inseminated with *Psammechinus* or *Paracentrotus* sperm. Pretreatment of the eggs with 0.5% crystalline serum albumin (Armour & Co.) increased the number of cross-fertilized eggs considerably. In the cross between *Arbacia* males and *Sphaerechinus* females mere removal of the vitelline membrane did not improve fertilization. If egg water of *Arbacia* was added, however, considerable cross fertilization occurred (10–65%) in the eggs devoid of vitelline membrane, whereas the egg water had only slight effect when the eggs retained their vitelline membrane.

Eggs in which the vitelline membrane had been removed by means of isotonic urea gave a higher per cent of cross fertilization with *Arbacia* sperm than eggs pretreated with trypsin. Addition of serum albumin (0.1%) or rabbit serum of 1/40 dilution increased the percentage of cross fertilization still more. The following outline shows the conditions which will give the highest cross fertilization with *Arbacia* males and *Sphaerechinus*, *Psammechinus*, or *Paracentrotus* females:

I. Removal of vitelline membrane.

II. Addition of *Arbacia* egg water.

III. Addition of 0.1% crystalline serum albumin.

Even if treatment I alone gives no effect, it is a prerequisite for the action of II and III.

The naked cytoplasmic surface is able to react with the spermatozoon and apart from the absence of a continuous membrane the fertilization is unimpaired. The species specificity is, however, less than it is in the presence of a vitelline membrane. This evidently is the carrier of the finer specificity of sea urchin eggs (117,118). It may thus have a selective effect on the sperm so that only those with the fitting complementary structure will penetrate.

In experiments with crystalline trypsin there is an upper limit to the concentration which may be used. After 30–40 min. exposure of *Psammechinus miliaris* eggs to a solution of crystalline trypsin containing more than about  $1.5 \times 10^2$  hemoglobin units per ml. (3), fertilization by spermatozoa of the same species failed to occur (238). Thus a protein localized in the cortex is sensitive to trypsin. The tryptic digestion of this protein blocks fertilization. This recalls the sensitivity of fertilizin to trypsin.

The surface of the spermatozoon is extremely resistant to trypsin. Treatment with 1% trypsin (Kahlbaum) over a five hour period still left the spermatozoa agglutinable by fertilizin (115). Wicklund (300) found that the fertilizing capacity of diluted sperm suspensions was not decreased by exposure to the crystalline trypsin in sea water. On the contrary, trypsin had an effect similar to that of albumin (see Sect. II). The period of high fertilizing capacity was prolonged. After two hours, for example, a sperm suspension fertilized only 10% of added eggs, whereas 90% of the eggs were fertilized if  $0.5 \times 10^2$  hemoglobin units of crystalline trypsin per milliliter of the suspension were present.

The result that addition of egg water facilitates cross fertilization invites speculation about the possible mode of action of fertilizin. Gray (84) found that addition of egg water to sperm suspensions delayed the decline of respiration. It may, for example, change the curve representing the rates of respiration from the type 1 or 2 to type 4 or 5 of Figure 2. In view of the correlation prevailing between respiration and capacity of fertilization one must predict that the egg water also prolongs the fertilizing life of the sperm. Egg water seems to act in the same way as the serum albumin in Wicklund's experiments. Hayashi (100b) obtained results with *Arbacia lixula* which seem to contradict those of Gray, but they were probably due to the strong agglutination of the *Arbacia* spermatozoa in egg water.

It is true, as pointed out by Tyler (272) that in general heavier sperm suspensions are needed to fertilize eggs deprived of their jelly coats than those with the jelly coats retained. It is doubtful, however, if this is valid for very fresh sperm suspensions. The curve of decline is, however, steeper when the eggs are without jelly. This means that the spermatozoa are stimulated when the eggs are surrounded by their jelly coats. A stronger suspension means a delayed decline of vitality of the spermatozoa and as a consequence also a greater capacity of fertilizing the naked eggs where the stimulating action of the jelly fails to occur. According to Wicklund (300) addition of serum albumin may render a sperm suspension capable of fertilizing nearly 100% of eggs deprived of their jelly coats even if the suspension in normal sea water gave a low percentage of fertilization of the naked eggs. Here albumin changes the curve of decline to a type with a prolonged period of high vitality like curve 5 of Figure 2. Under these conditions the spermatozoa are able to fertilize the naked eggs despite absence of the stimulating action exerted by the jelly. A merely stimulating action would tend to abbreviate the fertilizing life of the spermatozoon by exhaustion. Both the albumin and jelly coat substance, however, not only stimulate but also prolong considerably the period of high activity and fertilizing power. (ATP, on the other hand, stimulates the activity but hardly prolongs the period of activity.) In further research about these questions time curves should always be plotted. They will give a better clue than arbitrarily chosen points. Nothing can be said with certainty about the mode of action of albumin or jelly coat substance. As already intimated they serve possibly as nutrient material. Similar but less pronounced effects were in fact obtained with sugars (see Sect. II). A possible role of the active gelatin-liquefying enzyme present in the sperm (see above), may be to break down the protein component of the jelly coat fixed on the surface of the spermatozoon. This fixation is probably species specific as seems to follow from Hultin's results (118). Only the egg water from *Arbacia* favored the incorporation of *Arbacia* sperm with eggs of foreign species.

Many data thus point to the conclusion that the jelly coat or fertilizin may serve the purpose of maintaining a high vitality of the spermatozoon. According to Tyler 277, p. 199), an interaction between sperm antifertilizin and fertilizin drags the spermatozoa

nearer to the egg surface, through contraction of the micelles of the jelly coat. This writer believes that the spermatozoa of sea urchin penetrate the jelly coat by active movements and doubts that conditions prevail which would render the mechanism assumed by Tyler possible. In mature eggs a liquid space is present between the inner limit of the jelly coat and egg surface. The mechanism assumed by Tyler would rather impede the sperm in traversing the liquid space in which the spermatozoa show a high motility.

## V. Structural Changes Following Fertilization of the Egg

No general analysis of the structural changes following fertilization can be made at present. What takes place in the sea urchin egg will be considered primarily, but comparisons with other material will be attempted where feasible. As already mentioned, a contraction of the egg surface occurs upon attachment of the spermatozoon. The extent of the contraction varies according to the state of maturation of the egg cell (see Sect. II). This contraction of the egg surface seems to be of rather general occurrence (see 10,58,204,216) and it probably has to do with the expression of substances from the cortical layer of the egg. This is very obvious in the sea urchin egg, in which "cortical granules" are exuded. According to evidence presented by Runnström and co-workers (194,236,238,239,248,249) the cortical granules embedded in a ground substance merge with the vitelline membrane. Thus the final fertilization membrane has a dual origin. Of special interest is the transformation of the cortical granules into birefringent rods; this was particularly demonstrable when the merging of the granules with the vitelline membrane was delayed (236,238). Under these circumstances it was often observed that the exuded cortical material appeared in the form of distinct globules, each containing a certain number of cortical granules. These globules probably constitute the form in which the cortical material is normally exuded (see Figs. 3 and 4).

The vitelline membrane does not present any birefringence. The body of data appeared to indicate that it is constituted of two main components: the more complicated proteins which are responsible for the high specificity demonstrated in the experiments of Hultin (117,118), and the less complicated, irregularly arranged

fibrous proteins to which the high sensitivity to trypsin is due (247). During its elevation the membrane has particularly low resistance

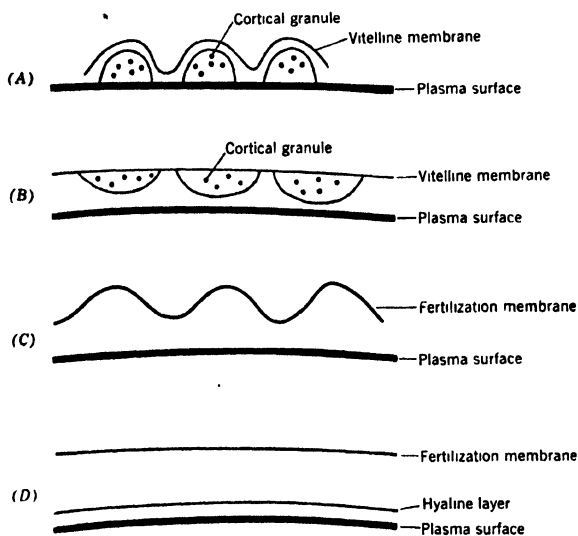


Fig. 3. Different steps in formation of fertilization membrane in fully mature eggs (236).

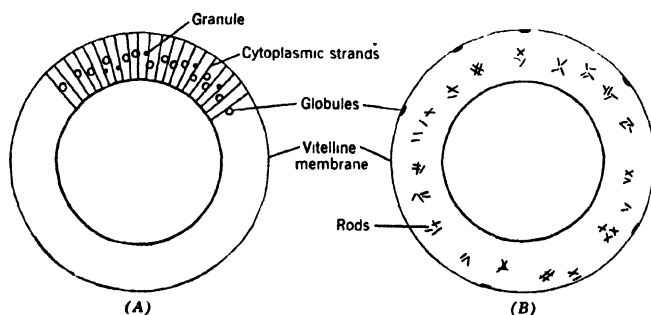


Fig. 4. (A) Delayed migration of granules and globules toward vitelline membrane. (B) Transformations of the granules into rods, globule attached to vitelline membranes (238).

to mechanical forces and to the action of isosmotic urea or potassium chloride solutions. It is also very sensitive to a proteolytic enzyme which can be obtained from the blastula of the sea urchin

(126). The fully elevated membrane, on the other hand, is very resistant to mechanical forces and is not affected by the reagents mentioned. It presents a birefringence (226) which is negative in the radial direction, the retardation of the membrane amounting to 12–13  $\mu$  (247,249).

Not only the cortical granules but also the exuded ground substance in which the granules are embedded undergo a considerable change in submicroscopic structure, a change revealed by the appearance of the birefringence of the membrane. The eggs of *Echinocardium cordatum* are especially responsive to reagents which bring about membrane formation (249). When eggs of this species were exposed to comparatively low concentrations of chymotrypsin or trypsin (e.g.,  $2.6 \times 10^2$  hemoglobin units), a very thin membrane appeared around the egg. It was elevated from the egg surface to the same extent as a normal fertilization membrane and was connected with the egg surface by many thin strands. Globules or granules flowed along these strands toward the membrane, which grew steadily thicker through the delayed incorporation of cortical material. These observations tend to show that lower concentrations of trypsin or chymotrypsin may bring about elevation of the vitelline membrane, whereas higher concentrations of the enzymes dissolve the membrane (238).

When eggs of *Psammechinus miliaris* devoid of vitelline membranes were fertilized, numerous globules and granules were expelled from the cortex. These are partly scattered in the medium surrounding the egg, and partly they stick to the egg surface. Even in the absence of vitelline membrane the cortical granules may be transformed into rods (239).

Solidification of the elevated fertilization membrane must be due both to alterations in the configuration of the protein molecules and to the formation of new end-to-end and cross links between the molecules, i.e., clotting or coagulation (238). The fertilization membrane is scarcely attacked at all by trypsin or chymotrypsin. If, however, the membranes are treated with thioglycolic acid, they become soft (188) and can then be attacked by chymotrypsin (194). Probably disulfide bonds also contribute to the stiffness of the fertilization membrane. Many interesting analogies present themselves when the changes in the fertilization membrane are compared to the observations of Ferry *et al.* (61,62) concerning fibrin.



Sometimes in the eggs of *Arbacia lixula* only a soft membrane is formed. It has the character of a fine gelatinous rim continuous with the surface of the egg cytoplasm. The formation of the gelatinous rim probably corresponds to what Heilbrunn (101) has designated "membrane swelling". This process corresponds to an incomplete membrane formation arrested in an early stage. The cortical granules have entered the membrane but have not been transformed into rods (see also above). In normal development the gelatinous rim stage passes rapidly. In the eggs of certain females, however, the gelatinous rim stage could last two to three minutes. Thereafter one or several local contractions were observed on the surface of the egg. Above the contracted areas the membrane separated from the cytoplasmic surface, elevated, and hardened. Usually the process spread gradually over the whole surface of the egg (241). This and similar observations (236, p. 13; 238, p. 4) point to the conclusion that a substance secreted from the egg cytoplasm incites the changes involved in the elevation and solidification of the membrane.

A water extract of lyophilized unfertilized *Arbacia* eggs was prepared and then dialyzed against sea water. Addition of this extract restored to eggs exhibiting only formation of the gelatinous rim in pure sea water the power of forming a normal fertilization membrane (250). It may be recalled that crystalline serum albumin and certain concentrations of ATP also did away with the inhibition of full membrane formation which prevailed in certain batches of eggs (see Sect. II).

Addition of basic protein from the spermatozoa (antifertilizin) of sea urchins or of clupein (0.002%) hinders the normal transformation of the cortical granules after fertilization (248,249). Crystalline lysozyme (0.025%) acts similarly (241), due probably to the basic character of this enzyme (179). In the presence of these substances the membrane remains only slightly elevated. Observation with the polarization microscope reveals lower retardation than under normal conditions. These changes may be due to a precipitating effect on the part of the basic proteins which prevents normal transformation of the cortical granules. Addition of iodocetamide (0.001 *N*) has a like effect (300b). This points also to the importance of sulfhydryl groups in formation of the fertilization membrane. In fact, it has been supposed that oxidation of

sulfhydryl groups to disulfide groups constitutes an important factor in the clotting of fibrinogen to fibrin (70). The formation of disulfide bridges is, however, probably not the only factor in the coagulation of the membrane-forming material. According to Brown (35), hydrogen bonds must play an important role in the mechanical properties of the fertilization membrane. Calcium is also necessary for coagulation of the membrane. If the eggs were transferred after attachment of the spermatozoon into calcium-free sea water and washed, a low granular membrane appeared (249).

In *Ascaris* a preformed vitelline membrane is also reinforced by the addition of material from below the protoplasmic surface. This material has a carbohydrate character and the membrane seems to consist mainly of chitin. After the formation of this membrane and the expulsion of certain vacuoles, a second membrane appears which is composed of a lipide, the ester of ascaric acid. Due to the secretion of material the egg diameter decreases from 60–70 to 45  $\mu$  (58).

In eggs of *Fucus spiralis* and *F. vesiculosus*, Levring (136) found a membrane formation mechanism which strikingly recalls the observations on sea urchin eggs. The vitelline membrane is thin and isotropic. Upon insemination a strong, birefringent fertilization membrane appears containing a central lamella of cellulose. Cortical granules are present in the unfertilized egg. These are squeezed out along with other material and give rise to an important part of the definite fertilization membrane. In *Nereis*, a jelly is forced out from the cortical zone of the egg which penetrates the vitelline membrane. The latter then separates from the egg surface (50,137, 202). In the egg of the fish *Oryzias latipes*, the contents of the cortical alveoli are forced out during fertilization. The initially soft membrane gradually hardens. Calcium was shown to be necessary if fertilization were to follow the normal course (304,305).

The surface covering the cortex of the mature unfertilized sea urchin egg (Fig. 1) looks like a luminous yellow layer in darkfield. Upon attachment of the spermatozoon, the color changes to white (224,226). The eggs of *Psammechinus* and *Echinocardium* are especially suitable for study of the color change, but it may also be observed in other species (211). The luminous layer is easily broken down by bee venom, suggesting the conclusion that it corresponds to a superficial layer of phosphatides (203). The egg surface

presents a birefringence which is positive in the radial direction (186,191,192,247). Treatment of the eggs with lipid solvents decreases the birefringence and may even change its sign. It is therefore probable that the structure of the egg cortex is such that radially arranged layers of lipid molecules alternate with tangentially arranged protein foils. According to Runnström, Monné, and Broman (247) the birefringence of the egg surface does not change upon fertilization of eggs from *Psammechinus miliaris* and *Echinocardium cordatum*. Conversely, Monroy (186) and Monroy and Montalenti (193) working with *Ps. microtuberculatus* found that the cortical birefringence disappears immediately after fertilization to reappear briefly for a certain period before segmentation of the egg. A more inconstant period of slight birefringence was also noted fifteen to twenty minutes after fertilization. Hypertonic treatment, however, makes the birefringence evident. The decay of birefringence following activation may be due to an increase in the distance between the molecules of the cortical layer without loss of orientation (193). This assumption makes the apparent difference between closely related organisms more understandable.

Observations with darkfield illumination also reveal changes in the interior of the egg. These are particularly obvious when eggs with a cloudy endoplasm are chosen for observation. Upon fertilization the ground substance of the cytoplasm becomes much darker (227).

Of special interest are the considerable changes which occur in the egg cytoplasm when females of *Ps. miliaris* are transferred to sea water with a 20–30% higher concentration than the normal medium (225,227,244). Monné (182,183) has investigated these changes further (see also 184, p. 9). The fibrillar (spongioplasmic) part of the cytoplasm is concentrated mainly in the center of the egg, while the vitelline material collects in an exterior zone. The mitochondria are also squeezed out from the condensed central part of the egg and are found on the border between the two zones. Birefringent fibrils are often observed here also. The material condensed in the central zone of the egg presents irregular patches or bundles of birefringent material. In extreme cases a polarization cross appears which is positive in the radial direction. This means a relatively complete orientation of the fibrils in a tangential direction. Only a low percentage of these eggs is fertilizable, but after

addition of 0.2% serum albumin to the sea water 100% fertilization may be obtained. The interior precipitation is now reversed and the structure of the cytoplasm becomes much more homogeneous even if the eggs remain in the slightly hypertonic medium. From these results the writer inferred that activation of the egg brings about dispersion of the cytoplasmic structure, which has a tendency to coarsen in the mature unfertilized egg. The cause of this change would be the more hydrophilic character of the colloids in the fertilized as compared to the unfertilized eggs. The observations with darkfield illumination referred to above are in keeping with these conclusions.

Even under observation with ordinary brightfield illumination the unfertilized eggs appear more opaque than the fertilized, indicating a stronger light scattering in the former. These differences are well reflected in the pictures obtained of appropriately fixed and stained preparations (see, e.g., 227, Fig. 2). Monné (185a) found recently that normal eggs of *Paracentrotus lividus* also present an obvious difference with respect to endoplasmic structure when ripe unfertilized and freshly fertilized eggs were compared. In the former the yolk granules are more irregularly distributed, whereas in the latter they are dispersed in a perfectly uniform way. The different distribution of the yolk granules no doubt corresponds to differences in the submicroscopic structure, this being coarser in the unfertilized than in the fertilized egg. The darkfield observations referred to above showed that the endoplasmic structure changes immediately follow the color change of the cytoplasmic surface.

It is of great interest that Pincus' observations on the rabbit egg fully agree with those above. Upon fixation the cytoplasm of unfertilized rabbit eggs has a rough, coarsely reticular appearance, whereas normally activated eggs have a uniformly granular cytoplasm (216, p. 55). The differences between unfertilized and fertilized sea urchin eggs are intensified if before fixation the eggs are exposed to a hypertonic solution (2 ml. sea water + 0.6 ml. 2.5 N sodium chloride) (see Sect. VI, where analogous experiments with artificially activated sea urchin eggs are referred to).

One may also express the facts referred to above by saying that the crystallization forces act differently in the unfertilized and the fertilized egg. In the former there is a tendency toward formation of numerous irregular aggregates (227, Fig. 11). Upon prolonged

subtraction of water a uniform tangential order of the fibrous elements may be brought about. In the fertilized eggs the structure is more homogeneous but an ordered aggregation occurs around the centrosomes. The fibrous elements have here a radial orientation.

This writer (241) observed that spontaneous activation sometimes occurs in unfertilized eggs of a smaller form of *Psammechinus miliaris* dredged from greater depths than the common litoral form. It was seen that tangentially directed fibers were present below the cortex. Gradually these fibers arranged themselves more radially, the proximal end pointing to the nucleus and the distal end still adhering to the cortex. The result was the formation of a monaster around a centrally situated, growing nucleus.

The colloidal state of the oocyte recalls in many respects that of the fertilized egg (190). The changes in the state of the colloids which occur after maturation may be correlated in some way with the decay of the egg centrosome which is generally believed to occur after the last meiotic division.

According to a theory emphasized particularly by Heilbrunn, activation means gelation of the egg protoplasm (101) (see also numerous references in the reviews, 102,103). According to Heilbrunn, the gelation is due to a clotting process suggestive of the clotting of blood. The experimental evidence for Heilbrunn's contention is provided by determinations with the centrifuge method of the viscosity of the cytoplasm. A very marked increase in viscosity follows fertilization. The viscosity of the interior of the fertilized egg is, however, subject to cyclic changes in the course of mitosis (66; 102, Figs. 14 and 15).

Runnström (227) has pointed out that the angular plasmolysis first observed by R. S. Lillie (146) in the fertilized egg may well be due to an increase in the viscosity of the cortex. The eggs were immersed in a hypertonic solution (2 ml. sea water + 0.6 ml. 2.5 *N* sodium chloride) at different times after fertilization. Immediately afterward they showed "convex" or smooth plasmolysis, but after five to six minutes they became increasingly angular. During a short period preceding segmentation they plasmolyzed again with a smooth surface. In the periods of smooth plasmolysis the viscosity of the cortex is probably lower. In the absence of calcium plasmolysis is smooth regardless of the stage. Hobson (108) claimed that angular plasmolysis was due to the properties of the

hyaline layer, but the cortex may be angular even when the hyaline layer is separated from the egg surface. This shows that angular plasmolysis depends on the properties of the cortex, and furthermore that these are very dependent on calcium.

Dan and Dan (54) found that certain pigmented granules are located directly beneath the cytoplasmic surface of the unfertilized egg of *Strongylocentrotus pulcherrimus*. By an inward migration of the pigmented granules an "extragranular zone" forms upon fertilization; the zone width is  $1.5\ \mu$ . According to Runnström and Monné (246), a cortical zone devoid of coarser granules is generally formed in sea urchin eggs upon fertilization. The change must be demonstrated on fixed and stained material. Exposure of the eggs to hypertonic medium prior to fixation further accentuates the presence of the extragranular zone in the fertilized eggs. In confirmation of Dan and Dan, it was also observed that the zone is absent in the parts of the cleavage cells contiguous with other cells. It seems very likely that the inward migration of the granules is connected with an increase in the viscosity of the cortex. Generally there is an expulsion of inclusions from cytoplasmic areas of increased density. Astrospheres, for example, appear as dark spots in observations with darkfield illumination. The mechanism may be the same as that which causes extrusion of impurities from a settling layer of virus particles in the experiments of Bernal and Fankuchen (18).

Mirsky's work (181) on the proteins from unfertilized and fertilized eggs of *Arbacia punctulata* and *Strongylocentrotus purpuratus* constituted an important step forward. The eggs were frozen, dried, and then extracted with cold 1 *M* potassium chloride solution at a pH of about 7.3. Between 82–85% of the total protein of unfertilized eggs passed into the solution; after fertilization, however, only 67–69% of the protein dissolved. Thus a certain fraction became insoluble. This fraction first is salted out by means of ammonium sulfate from solutions of proteins from unfertilized eggs and exhibits high viscosity and double refraction of flow.

It is evident that a sort of coagulation of the "Mirsky protein" occurs in connection with fertilization. This coagulation begins about four minutes after insemination and is complete within the next ten minutes. Thereafter the coagulated state remains constant until two hours after insemination, i.e., as far as the research was

extended. Connors and Scheer (44) have continued Mirsky's work; they found that the "Mirsky protein" forms an electrophoretically uniform fraction; its light absorption does not indicate the presence of nucleic acid. The "Mirsky protein" does not exhibit adenosine-triphosphatase (ATPase) activity, although its properties recall myosin.

Coagulation of the "Mirsky protein" probably accounts for physicochemical changes which occur in the egg and particularly in its cortex upon fertilization. The time schedule of the progress of coagulation corresponds almost exactly with the development of the cortical changes as manifested by formation of an extragranular zone and the appearance of angular plasmolysis in a hypertonic medium.

Studies have been carried out in the writer's laboratory by Monroy (190a) on proteins extracted from lyophilized eggs of *Paracentrotus lividus* by means of distilled water. As there are sea-water salts left in the material this amounts in reality to extracting with weak salt solutions. The extracts have given rather clear electrophoretic patterns. They contain at least five components, which all migrate toward the anode. Two components, 1 and 2, are fairly large; they are precipitated by ammonium sulfate above 50% saturation. A small component, 3, migrates more rapidly than 1 and 2. A further small component, 4, migrates much more slowly than 1 and 2. Between 4 and 1 a component 5 appears, which never separates very well. Upon fertilization certain changes occur with respect to the electrophoretic pattern. An apparently new component, 6, appears between 3 and 2. The solubility of fraction 1 undergoes a change, so that it is precipitated by ammonium sulfate already at 50% saturation. Component 6 disappears again so that it is not visible in extracts from eggs frozen 30 minutes after fertilization. On addition of 0.1 *N* sodium chloride to the buffer solution the two main components, 1 and 2, merge into one single peak. This effect is reversible when the sodium chloride is removed by dialysis. Potassium and lithium chlorides do not change the electrophoretic pattern obtained in the buffer solution (phosphate buffer: ionic strength 0.1; pH varied, but in general 6.83).

The electrophoretic pattern of water extracts from eggs of *Arbacia lixula* is somewhat different from that of *Paracentrotus lividus*. The concentration of component 2 is lower in *Arbacia* than in *Paracen-*

*trotus*. Two faster components, 3 and 6, are present before fertilization, but following this they merge into one larger component.

According to Mirsky (181), unfertilized eggs disintegrate when frozen and thawed, whereas this treatment does not break up fertilized eggs. Upon transfer to strongly diluted sea water unfertilized eggs tend to disintegrate into numerous spheres and granules, whereas a fibrillar framework persists in fertilized eggs even after considerable amounts of substance have been dissolved (227). Particularly, the cortex of fertilized eggs exhibits a complicated fabric. Previous removal of the fertilization membrane does not change the picture. The more intimate linkage of the colloids of the fertilized as compared to the unfertilized egg is demonstrated by this apparently rough experiment.

Under certain experimental conditions the break-up of the unfertilized eggs may assume a type more like that characteristic of the fertilized eggs. It is of particular interest that according to observations of Wicklund (300) pretreatment of unfertilized eggs with 0.001 to 0.002 *M* ATP produces this effect in eggs of *Arbacia lixula*. After great dilution of the sea water with distilled water precipitation of the jelly coat is observed above a point on the surface of the egg where an outflow of endoplasm has occurred. If the eggs have been pretreated with ATP, however, a strong precipitation of the jelly coat occurs all around the egg. The vitelline membrane appeared tougher than in eggs not treated with ATP. This was particularly evident if the eggs were first divested of their jelly coats. The ATP-treated eggs proved to be more resistant to hypotony than the control eggs. ATP favors coagulation processes in the cytoplasm, the membrane and the jelly coat. Its improving action on the fertilization membrane formation (see above) is probably due also to a coagulative effect.

Cephalin becomes more firmly bound to the proteins after fertilization of the *Echinocardium* egg (203). Lindberg (155) provided evidence that a greater part of the glycogen is bound to proteins in the fertilized than in the unfertilized egg. These data concur to show that lipides and carbohydrates combine more readily with proteins in the fertilized than in the unfertilized state.

According to Lillie (144,145) an increase in permeability to water follows upon fertilization. Stewart and Jacobs (262) demonstrated further increased permeability to anelectrolytes like ethylene gly-



col. These changes in permeability undoubtedly also reflect changes in the fine structure of the protoplasmic surface.

## VI. Artificial Parthenogenesis

In the writer's opinion R. S. Lillie's hypothetical considerations concerning the mechanism of artificial parthenogenesis are the most consistent so far presented. They are based mainly on numerous experiments on starfish eggs. A review and discussion of most of this work has been presented by Lillie (147). Starfish eggs may be activated by treatment with an 0.004 *M* solution of butyric acid for six to seven minutes. After being returned to sea water, the eggs, in a high percentage of cases, develop into free swimming blastulas. Warming of the eggs at 32° for five to six minutes has the same effect. Acid or heat treatment of the eggs may be interrupted after a time and completed later. Initial treatment with acid may be supplemented by warming, or heat treatment may be supplemented by acid. This seems to indicate that a certain reaction product is gradually accumulated and that development ensues only when this product arrives at a certain level of concentration. The evidence points further to the conclusion that the same product is formed subsequent to exposure both to acid and to elevated temperature. Both agents are active even in the absence of oxygen. Briefer treatment of the eggs brings about a partial activation involving, for example, membrane and monaster formation but no cleavage. This more incomplete activation has been thoroughly considered by Dalcq, Pasteels, and Brachet (53). Thus it seems that certain processes occur at a lower level of accumulation of a product, whereas complete activation follows only upon a higher degree of accumulation within the egg.

The eggs of *Asterias* may also be activated by exposure to a hypertonic solution, *e.g.*, 100 ml. sea water + 10–30 ml. 2.5 *M* sodium–calcium, *i.e.*, 95 volumes 2.5 *M* sodium chloride plus 5 volumes 2.5 *M* calcium chloride. After exposure for ten to thirty minutes at 20° and return to sea water, the eggs form membranes and pass through some stages of segmentation. If, however, after fifteen to twenty minutes in sea water the eggs are treated with 0.004 *M* butyric acid in sea water, they will develop into a free swimming stage after return to normal sea water. The previous exposure to

hypertonic sea water considerably abbreviates the optimum time of treatment with acid (or warming). This means that the actions of hypertonicity and of acid (or warming) are additive. According to Lillie, however, the character of the action of the hypertonic solution differs from that of acid or warming. The first-named is efficient only in the presence of oxygen. Lillie interprets this as an indication that two substances are necessary to effect activation. One of these is assumed to be a synthetic product, *S*, the formation of which is favored in a hypertonic solution in the presence of oxygen. The second substance, *B*, is, according to Lillie's contention, the product of a splitting process which is independent of oxygen. The process is favored by exposure to acid or increased temperature. The final activation would correspond to the reaction  $S + B = A$ , where *A* is the activating substance.

In Loeb's (163, p. 65) "improved method" of parthenogenesis, sea urchin eggs (e.g. of *Strongylocentrotus purpuratus*) are treated with butyric acid for some minutes and then returned to sea water. After an interval of fifteen to twenty minutes the eggs are put in a hypertonic medium (50 ml. sea water + 8 ml. 2.5 *N* sodium chloride). After fifteen to sixty minutes the eggs are placed, at five minute intervals, in normal sea water. The eggs exposed to the hypertonic medium for the appropriate time cleave normally and develop. It should be noted that the eggs may be exposed to both treatments, even if the order is reversed, with the same outcome. Loeb concluded from his results that the activation has a dual character. One factor—represented in his experiments by the fatty acid—brings about an incipient cytolysis of the surface layer of the egg. A second factor exerts a correcting influence which prevents the impending cytolysis and directs the development into normal paths. In Loeb's experiments the exposure to a hypertonic solution serves as the correcting factor.

When Loeb's method is used treatment with fatty acid brings about cortical changes and induces nuclear growth and cytoplasmic radiation centered on the nucleus, the monaster. Chromosomes are formed and the nuclear membrane disintegrates but is formed anew without division of the nucleus. This process is repeated a number of times until it finally stops. In certain cases, however, cleavage may occur and proceed a few steps (107).

It is evident that the fatty acid treatment of sea urchin eggs is

capable only of incomplete activation of the egg. It must be supplemented by exposure to the hypertonic treatment. Lillie's views concerning the accumulation of two different substances seem to be applicable here also. These views are further supported by the fact that the hypertonic treatment may precede or follow fatty acid treatment.

Just (122) demonstrated that membrane formation and complete development may be obtained by one treatment with a sufficiently strong hypertonic solution. He is therefore opposed to a dual concept of the activation process. Lillie's concept unites these opposing views. His concept involves assumption of the dual origin of a unitary factor which when present in sufficient concentration induces complete development.

Bataillon (15) explained the action of the hypertonic solution in Loeb's improved method by assuming that it co-ordinates the rhythms of the nuclear changes with those of the aster and spindle formation to produce normal interaction between these somewhat independent events. Treatment with hypertonic solution for an appropriate period helps the egg to produce a dicentric spindle that brings about regular distribution of the daughter chromosomes.

Bataillon (15) confirmed Just's (122) results concerning the activating effect of strong hypertonic solutions. He stressed the fact that these solutions must be alkaline. Thus according to Bataillon two factors are involved, the hypertonicity of the medium and its alkalinity. Furthermore, Bataillon demonstrated that the addition of potassium cyanide improves the results. This addition entails the suppression of accessory astrospheres which tend to disturb the normal course of cell division.

Studies of artificial parthenogenesis in frog eggs seem to support the theory that there are two steps in the activation process. Bataillon (13,14) used the method of pricking the eggs with a glass needle. Mere pricking, however, tends mainly to bring about cortical changes which may entail a number of cell divisions but no further development. As shown by Bataillon, more regular cleavage and further development occurs if inoculation of white blood cells is combined with the pricking (see also the earlier work of Guyer, 87). The inoculation of the blood cells incites formation of a cytaster. Without forming a component of the division figure, the cytaster assists in the formation of the bipolar spindle and in the cytoplasmic

division. On the basis of a unitarian concept of activation the formation of a dicentric spindle may be regarded as the most exacting process with respect to accumulation of "activating substance" or its substrate. Nuclear activation seems to be less exacting.

Reverberi (220) carried out cross fertilization between two ascidians, *Ciona intestinalis* (female) and *Phallusia mamillata* (male) (see Sect. IV). In many cases the eggs were activated by mere contact with the spermatozoa, which did not penetrate the eggs, but on the contrary were detached from the egg surface. These eggs presented a monaster formation but no segmentation. Normally extensive cytoplasmic currents bring about a rearrangement of the granular material of the egg of *Ciona*. In the incomplete activation observed by Reverberi only very slight movements occurred. Possibly, incomplete activation does not in general give enough impetus to bring about cytoplasmic movements, which may favor certain reactions by increasing the probability of contact between different substances.

The centrosome is regarded as the division organ of the cell, although it is not present in phanerogamic plants. Following the maturation divisions the egg centrosome disappears and is replaced in the fertilized egg by the centrosome of the spermatozoon. It seems very probable that the centrosome should be regarded as a self-perpetuating cell organ. Its character and mode of action is obscure, however. Boveri (28) assumed that activation of the egg involves the introduction of a new centrosome with the spermatozoon. In this way the egg reacquires its capacity for division. However, data like those presented by Reverberi for ascidian eggs prove that activation is effected by the mere attachment of the spermatozoon to the egg surface, as was first proved by Bataillon (11,12) and Lillie (137). An optimum treatment with parthenogenetic agents causes what is believed to be a regeneration of the egg centrosome. On the other hand it is certain that centrosomes may also form *de novo* in the egg.

The results obtained by Harvey (95-99) were especially significant. She used centrifuge tubes containing a bottom layer of isotonic sucrose with a layer of sea water above. The eggs accumulated on the border between these layers. Under the influence of the centrifugal field the eggs were elongated and finally broke into two parts, the lighter containing the nucleus and the heavier the

pigment, mitochondria, and vitelline material. The nonnucleated part could also be activated by treatment with a hypertonic solution. When returned to normal sea water these eggs could divide into a great number of cells. It is evident that the centrosomes arising here are able to divide, although in the absence of nuclei no spindle formation occurs. It is not probable that any of these centrosomes derive from the original egg centrosome. They have arisen *de novo* but are nevertheless self-perpetuating.

Hollaender (109) irradiated sea urchin eggs with ultraviolet light of different wavelengths. The optimum of activation was given by the short wavelengths below 240 m $\mu$ . This corresponds to the maximum absorption of proteins. The wavelengths corresponding to the absorption maximum of nucleic acid were not especially active. These results probably indicate that it is the proteins, not the nucleic acid, which are primarily involved in the activation process (see also 100).

Paspaleff (211) carried out a number of experiments in which the eggs of *Paracentrotus* were subjected to a solution of 10% magnesium bromide in sea water. The time of exposure varied between thirty and ninety minutes. The temperature was 16–18°. When the eggs were treated with hypertonic solution immediately after removal from the ovaries, development was very poor. Only nuclear activation was obtained. Then the same egg material was subjected to hypertonic sea water after having remained twelve hours in sea water of pH 7.4. Now the development was much improved, but still better development was obtained after the eggs had remained in sea water for 24 hours before transfer to the hypertonic solution. It is evident from Figure 5 that the period of exposure to hypertonic solution could be abbreviated as the age of the eggs increases. This proves that something has accumulated in the egg during its sojourn in sea water (see Sect. II). It can be inferred that the accumulating substance facilitates activation through the hypertonic solution.

It has been described above how the duration of the wrinkled state, which occurs soon after immersion of the eggs in a hypertonic solution of sufficient strength, decreases with progressing maturation. It may be inferred from Paspaleff's report that optimum development is obtained if exposure of the eggs to the hypertonic solution is prolonged until their surface becomes smooth. With

progressing cytoplasmic maturation the time required to reach the smooth stage is abbreviated (see Sect. II), and concurrently the optimum time of exposure to the hypertonic solution becomes shorter. Thus it seems that the smoothing of the eggs in the hypertonic solution constitutes a step toward activation.

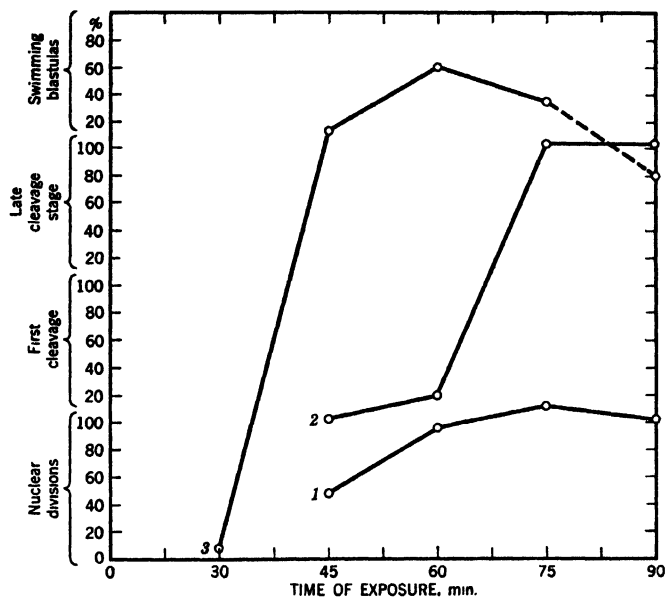


Fig. 5. Treatment of eggs of *Paracentrotus lividus* with 10% magnesium bromide in sea water (modified from Paspaleff, 211). Eggs treated (1) immediately after removal, (2) after storage for twelve hours, and (3) after storage for 24 hours in sea water, pH 7.4.

Runnström and Monné (246) found in experiments with *Psammarchinus* eggs that addition of potassium cyanide or azide to the hypertonic sea water (2 ml. sea water + 0.6 ml. 2.5 N sodium chloride) enhances attainment of the smooth state. The addition of these also shortens the period of exposure necessary to obtain activation. This is analogous to Bataillon's (15) experience of the favorable effect of an addition of potassium cyanide to strong hypertonic solution.

An increase in temperature increases the rate at which the eggs arrive at the stage of maturation in which smooth plasmolysis occurs upon exposure to a certain standard hypertonic solution. When the eggs were kept at 17°, smooth plasmolysis occurred in the hypertonicity test only after 22 hours (246). Eggs kept at 26.2° for two hours gave smooth plasmolysis after transfer to the hypertonic medium. The rate of the underlying change thus increased about ten times with an increase of 9°C. Some of the eggs tested proved to be already activated, since upon transfer to the hypertonic solution they exhibited angular plasmolysis. The number of activated eggs in the 26.2° sea water increased with prolonged exposure. Development of the eggs did not advance beyond the monaster stage. If the eggs were exposed to the higher temperature in calcium-free sea water, no activation took place. This is in agreement with data presented by Moser (198).

It is of interest to study the cytoplasmic structure of eggs transferred to a hypertonic solution. In Figure 6 sections of eggs are shown which were fixed in Bouin's fluid and stained with Heidenhain's iron hematoxylin. The structure of an egg treated with hypertonic solution (Fig. 6b) is coarser than that of a control in sea water (Fig. 6a). In the former the spongioplasm has contracted into numerous nodes around which the mitochondria are visible. The eggs just described had been kept at 17.8°. The picture was different when after three hours of exposure to 26.2° sea water the eggs were transferred to hypertonic sea water. The nucleus is found in the center of the egg; the cytoplasmic structure is fairly homogeneous (Fig. 6c). Below the cortex a layer of darkly stained mitochondria has accumulated. This is the same phenomenon as occurs in fertilized eggs (see Sect. V). The mitochondria were driven from the gelating cortex and accumulated in a layer situated immediately below the cortical layer. Figure 6d represents an egg which has also been subjected to a temperature of 26.2°, but in calcium-free sea water, and subsequently transferred to a hypertonic calcium-free medium. No cortical change has taken place. Most of all, the eggs recall the untreated control (Fig. 6a); they have not even reacted by becoming coarser, like the eggs transferred from normal sea water to the hypertonic medium (Fig. 6b). Closer examination reveals, however, that the fibrillar cytoplasmic structure in the eggs treated with calcium-free medium is more dis-

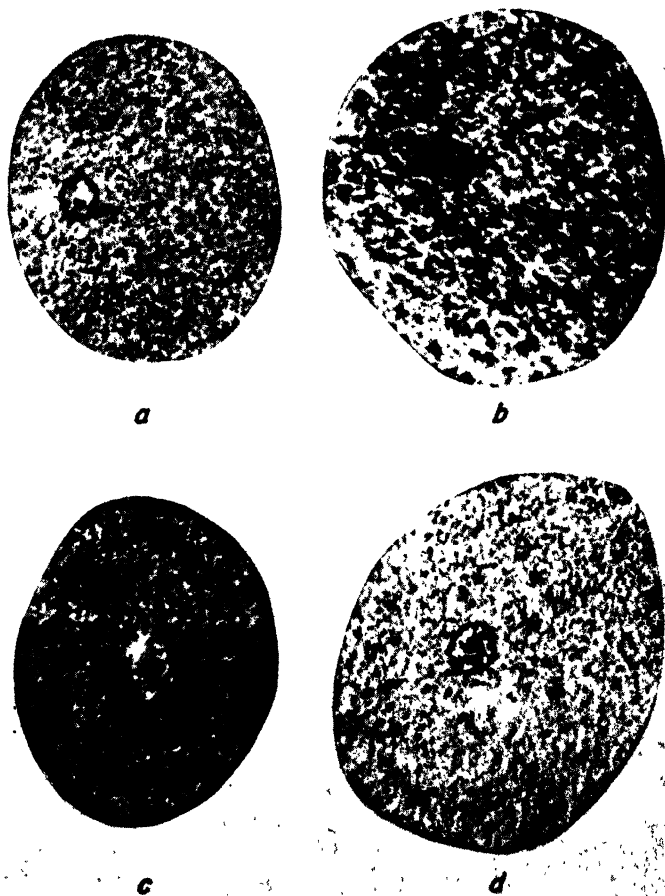


Fig. 6. (a) Normal mature egg. (b) Mature egg in hypertonic sea water previously kept for three hours in normal sea water at 17.8°. (c) Mature egg in hypertonic sea water previously kept for three hours at 26.2°. (d) Mature egg in hypertonic calcium-free sea water previously kept for three hours in calcium-free sea water at 26.2° (246).



tinct than in eggs kept in normal isotonic sea water. Furthermore, single large granules appear scattered over the whole cytoplasm. Thus these preparations demonstrate clearly that calcium is necessary to bring about the cortical gelation which appears in connection with fertilization or artificial activation.

Unfertilized eggs of different sea urchin species were subjected to the action of commercial trypsin, 0.1 to 0.4% in sea water. After thirty to sixty minutes almost 100% of the eggs showed nuclear activation. The nucleus migrated toward the center of the egg and a monaster appeared (249). Bohus-Jensen (20) did not find the same effect when working with crystalline trypsin or chymotrypsin but the problem of the activating effect of different proteolytic enzymes is under further examination in our laboratory.

Activation of sea urchin eggs also occurs, as found by Moser (199), when they have been treated with an isotonic solution of urea and then returned to normal sea water. Exposure both to commercial trypsin and to urea dissolves the vitelline membrane. This dissolution may be closely connected with activation. It was shown (see Sect. V) that exposure to a certain rather low concentration of crystalline trypsin or chymotrypsin causes elevation of the vitelline membrane (238).

Clupein dissolved in sea water (0.01 to 0.005%) causes activation of the eggs of *Psammechinus* (249,298). A low, more-or-less granular membrane is elevated. The nucleus migrates to the center. A monaster is formed and even some irregular divisions occur. It is of interest that antifertilizin from the sperm of sea urchins has the same effect as clupein. Thus substances extracted from sperm according to Hultin (115) have proved capable of inducing the first steps in development. The eggs must be divested of their jelly coats in these experiments, since otherwise the basic substances used precipitate the jelly coat (see Sect. III). Even the naked eggs agglutinate (65) but this does not impede activation. On the contrary, a certain correlation was found between the agglutination and activation of the eggs (249).

Lillie (147) found that treatment of starfish eggs with acid or elevated temperature was more efficient when the eggs were immersed in diluted sea water. The time of exposure could be considerably abbreviated. Tyler (273) found that eggs of *Urechis* could be activated by mere exposure to diluted sea water and

Harvey (99) made a similar observation concerning the eggs of *Arbacia*. Lillie interprets the action of diluted sea water as due to the greater rate of diffusion in egg cells which have swollen in hypotonic medium. This increased rate of diffusion, on the other hand, would facilitate the interaction of substances. A hypertonic solution acts in fact in the opposite way, as confirmed by Runnström and Monné (246) in the experiments mentioned above on the action of elevated temperature on sea urchin eggs.

In a number of publications the role of calcium in activation was particularly emphasized. Salt mixtures with a high concentration of calcium chloride induce, according to Daleq and co-workers, the development of eggs of the starfish *Asterias glacialis*, the worm *Phascolion strombi*, and the mussel *Barnea candida* (see 51,53,212, 213).

Similar results were obtained by Heilbrunn and co-workers. The eggs of the mussel *Cumingia* are activated when immersed in pure solutions of calcium chloride (110). The eggs of the annelid worm *Nereis* may be stimulated by ultraviolet radiation, but this activation fails to occur if the eggs have previously been immersed in sodium citrate solution. According to Heilbrunn and co-workers (103,104,301) activation is brought about by release of calcium in the cortical layer. Hultin (119) prepared homogenates of unfertilized eggs of *Psammechinus miliaris* in calcium-free sea water (see Sect. VII and Fig. 11). These homogenates exhibited a strong scattering of the light. On addition of calcium to the homogenates the light was less scattered; thus they appeared more homogeneous and transparent. This recalls indeed the changes taking place in the living egg upon activation (see Sect. V).

In eggs like those of *Cumingia*, *Asterias*, *Barnea*, and *Urechis*, where the activating agent is applied before the meiotic divisions, the latter may remain submerged and the presumptive polar spindles become cleavage spindles. If, however, the polar bodies form in a normal way, the activated egg is capable only of monaster formation. The submerging of the meiotic divisions—depolarization according to Daleq's terminology—may keep the egg centrosome from waning and thus these eggs probably have an intact division apparatus which allows them to undergo complete development. In activated starfish or *Urechis* eggs which perform a complete meiosis conditions prevail similar to those in the sea urchin egg.

In types like the sea urchin the fertilization occurs after the meiotic divisions, *i.e.*, in a stage when the egg centrosome has already waned. Therefore artificial formation of a dicentric division apparatus is a more exacting procedure here. The same holds true of the frog and toad egg in which submerging occurs only in exceptional cases (17). Pricking with the needle, any other activating treatment as electric shock or exposure to chloroform generally induce the expulsion of the second polar body (see 15, p. 712).

In the butyric-acid-treated sea urchin eggs or the pricked but not inoculated frog or toad eggs the anabolic processes may be incompletely activated. Synthesis of nuclear material occurs, but the self-perpetuation of the central bodies cannot be maintained on this level of activation. This is probably the effect of a general inertness of the synthetic processes in the cytoplasm. In normal fertilization the male central body suppresses any tendency to activity of the female. Inhibitory effects of similar kind were observed by Fankhauser and Moore (57).

The question of the depolarized or submerged meiotic divisions has been so thoroughly reviewed in the monographs of Daleq (52) and Tyler (273) that no further discussion is necessary here. For interesting discussions see also Daleq, Pasteels, and Brachet (53), and Pasteels (212,214). Concerning artificial activation in mammalian eggs see Pincus' monograph (216). Runnström (228) has reviewed the methods of parthenogenesis used in work with different animals. The main object of this chapter has been to show how a state of activation may gradually be built up in the unfertilized egg. The evidence points to the conclusion that a precursor present in the egg is gradually transformed into an active agent. Activation ensues when this agent has arrived at a certain threshold concentration. The threshold may, however, be different for different processes, thus explaining the oft-encountered incomplete activation. Cytoplasmic maturation is a comparatively slow spontaneous change of a kind like that brought about more rapidly by activating agents (see Sect. II). Transformation of the precursor may demand the presence of certain activators. Calcium may be one of these. It will be demonstrated below that in the sea urchin egg calcium certainly contributes to the activation of certain enzymes. The views expressed correspond in essentials to the conclusions already formulated by Lillie (147). Pasteels (212,213)

approaches a similar conception when he introduces the notion of "sensitizers" which prepare the egg for the action of the final "realizer."

## VII. Metabolic Changes Following Fertilization

Warburg (288,289) was the first to show that a considerable increase in oxygen consumption takes place upon fertilization or artificial activation of sea urchin eggs. This result has since been confirmed by a large number of workers. The quotient between the oxygen consumption in the fertilized and the unfertilized egg is somewhat variable. Ballentine (6) and Borei (25) have collected data from various authors for some sea urchin species; these data are given in Table IV. As is nowadays well known, sea urchins do

TABLE IV

Organism	QO <sub>2</sub> (fertilized)/QO <sub>2</sub> (unfertilized)			
<i>Arbacia punctulata</i> .....	2.6	3.9	4.4	4.5
	5.0	5.0	5.0	5.3
<i>A. lixula</i> .....	6.0	—	—	—
<i>Psammechinus microtuberculatus</i> ..	3.8	—	—	—
<i>Ps. miliaris</i> .....	3.6	5.7	—	—
<i>Paracentrotus lividus</i> .....	3.1	6.0	—	—

not represent a typical case with respect to changes in oxygen consumption following activation of the egg. Loeb and Wasteneys (166) found no difference in the oxygen consumption of fertilized and unfertilized starfish (*Asterias*) eggs. Much new data have been added by Whitaker (292-295), who has also given a comprehensive review of the field (296) (see also 34,200). In many cases there is little or no change in the rate of oxygen consumption; even a decrease has been noted, namely, in the annelid *Chaetopterus* (32,295). Older work seemed to indicate a certain increase in the rate of respiration of frog eggs, but more recent research by Brachet (31) and Stefanelli (261) has shown that no increase takes place. On the other hand Brachet was able to demonstrate a considerable decrease from 0.99 to 0.66 in the respiratory quotient of the frog egg after fertilization. This indicates a change in the character of the substrate oxidized (34, p. 116).

Lindhahl and Holter (154) have used the diver method of Linderstrøm-Lang (159,160; see also 111) to measure oxygen consumption of *Paracentrotus lividus* eggs. They found that the rate of respiration in the primary oocytes was slightly higher than that of the eggs after fertilization. After maturation, however, a considerable decrease in respiration ensues. If sea urchin eggs were fertilized in primary oocyte stage a slight decrease in respiration would be noted. Their exceptional position is due to the fact that they cannot be fertilized until completion of the maturation divisions.

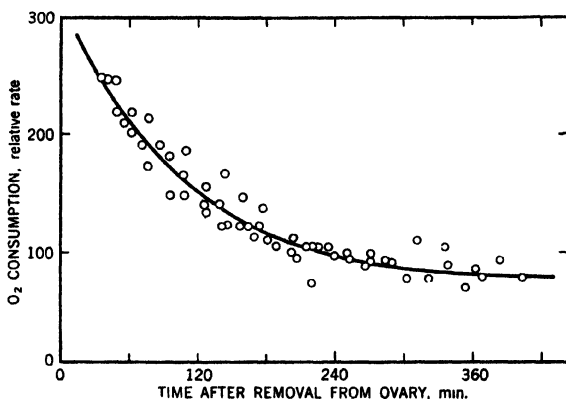


Fig. 7. Oxygen consumption in unfertilized eggs of *Psammechinus miliaris* as a function of time elapsed since their removal from the ovary (25).

Borei (25), working with the diver method on eggs of *Psammechinus miliaris*, arrived at results somewhat at variance with those of Lindahl and Holter (154). He found that the rate of respiration of the unfertilized eggs is a function of the time elapsed since the removal of the eggs from the ovary (see Fig. 7). The curve can be interpreted to mean that two respiratory systems are present in the unfertilized egg, one declining exponentially and the other constant. An asymptotic value of the respiration is obtained when the declining component approaches zero. The curve of Figure 7 recalls what is known about the course of the endogenous respiration of baker's yeast (22,263). The asymptotic value has been made equal to 100 in the figure. The first part of the curve must be

extrapolated, as experimental conditions did not permit measurement to be begun less than forty minutes after removal of the eggs from the ovaries.

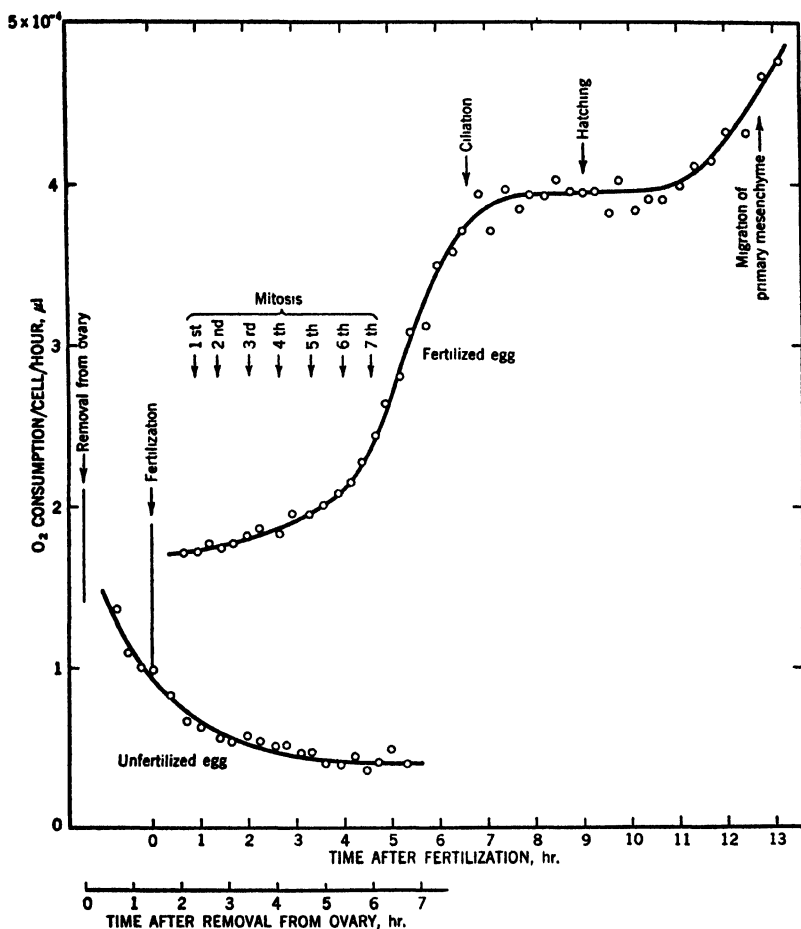


Fig. 8. Respiration of *Psammechinus miliaris* egg from fertilization until early gastrula stage (25).

In Figure 8 the curve of Figure 7 is represented together with a curve for the respiration of the eggs from the moment of fertilization

until the stage of immigration of the primary mesenchyme. The level of respiration attained after fertilization is the same irrespective of the moment of fertilization (27). This means that, if fertilization occurs rather soon after removal of the eggs from the ovary, the respiration may be even higher or at least as high in the unfertilized as in the fertilized eggs. The longer the lapse of time after removal of the eggs, the greater the increase in respiration following fertilization, until finally, after attainment of the bottom value of respiration in the unfertilized eggs, the increase must be constant. Under natural conditions the eggs are probably fertilized immediately after spawning, which means that no increase, or rather, a slight decrease in respiration takes place.

Borei (25) found a similar although slower decline in the respiration of oocytes or mature eggs of *Asterias glacialis* (see Fig. 9).

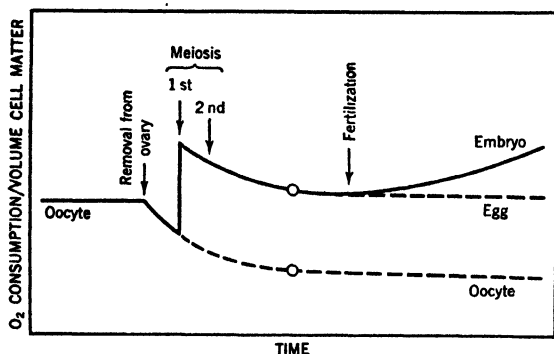


Fig. 9. Oxygen consumption of *Asterias* in early phases of development (25).

At the first meiotic division there is a sudden increase in respiration, but fertilization does not cause any change (see, however, 257). Results similar to those obtained by Borei with sea urchin and starfish eggs were reported earlier by Holter and Zeuthen (112) for unfertilized eggs of *Ciona intestinalis*. Their curve (112, Fig. 7) is of the same type as that reproduced here in Figure 7. No increase in respiration takes place upon fertilization.

The rate of respiration in fertilized sea urchin eggs (Fig. 8) increases exponentially (81,151). Lindahl (151) found that after

the "exponential" period the curve arrives at a plateau, but that shortly before the first sign of gastrulation the rate of respiration begins to increase again, although now as a linear function of time. In the starfish egg also an exponential increase in respiration of exactly the same type as that in the sea urchin egg begins after fertilization (Fig. 9). (For a more complete curve see 26.) An exponential increase in respiration is also found in *Chaetopterus* (295) and *Ciona* (112). The curves in Figures 8 and 9 suggest that the point of general interest is not whether or not a sudden change occurs at the moment of fertilization; it is more important that fertilization induces a gradual increase in the rate of respiration.

Zeuthen (306) has demonstrated cyclic variations of respiration in developing *Psammechinus* eggs. The respiration is higher in the prophase, and declines somewhat in the later phase of each mitosis. The writer recognized these minor variations earlier (232) in the first mitosis of the sea urchin egg. The variations do not appear in the over-all curve. It is tempting to believe that the increase is due to the synthesis or release of some limiting link in the respiratory system. It is of great interest that the increase in respiration is only slightly lower in syncytia formed by reversal of cleavage in *Ciona* eggs than in normal larvae of corresponding age. Thus the formation of cell boundaries and the differentiation are not essential to the gradual increase in respiration following fertilization (2). In this connection one may recall Warburg's (289) results concerning the effect of phenylurethan on fertilized *Paracentrotus* eggs. In the concentration used (0.002 *N*) the drug does not decrease respiration, but it does impede cytoplasmic segmentation. The synthesis of nuclear substances, on the other hand, goes on.

Gray (81) and Lindahl (149,151) recognized two components in the respiration of the fertilized sea urchin egg, one constant and one increasing. From the increase in the respiratory quotient Lindahl inferred that the latter component results from an oxidation of carbohydrates. Fisher and Henry (64) recognized two components of the respiration in the fertilized *Arbacia* egg, one sensitive and one insensitive to urethan.

In certain animals, such as the frog and toad, the rate of respiration seems to be rather constant during the first phases of development (31,261) and the gradual increase occurs only at a later stage. In these organisms segmentation may also proceed under con-



ditions of anaerobiosis, whereas the segmentation of sea urchin eggs stops in the absence of oxygen.

Loeb (163, p. 281) pointed out that respiration accelerates the death of the unfertilized egg. "The fertilization saves the life of the egg." If unfertilized starfish eggs are kept under anaerobic conditions, their life is prolonged. Loeb maintains that the earlier death of the aerobically kept eggs is not due to bacterial contamination. It is now clear that the eggs were not in strictly anaerobic state in Loeb's experiments (7). At any rate, it would be of great interest to inquire which inhibitor may be formed through oxidation processes in the unfertilized egg. Apparently these products are not formed or are more quickly removed in the fertilized egg. In this connection the work of Tosic and Walton (267-269) on bovine sperm may be recalled. They demonstrated the aerobic formation of hydrogen peroxide in living spermatozoa in connection with the dehydrogenation and deamination of some cyclic amino acid. Loeb's observations on starfish eggs may possibly reveal a qualitative difference between the quantitatively equal oxidation processes in the unfertilized and the fertilized starfish egg.

The increase in the rate of respiration following activation of sea urchin egg seems in the light of newer findings to be a rather special case. Moreover, this increase probably does not even occur under natural conditions. Nevertheless, the phenomenon is of great general interest and further efforts toward its elucidation seem justified. The writer (229) has demonstrated that the respiration of the fertilized egg of the sea urchin *Paracentrotus lividus* is inhibited in mixtures of carbon monoxide and oxygen, whereas the respiration of unfertilized eggs is not inhibited. On the contrary, an increase in the rate of oxygen consumption is observed in the unfertilized egg in the presence of carbon monoxide. The enhancing effect prevails indeed in the fertilized egg also, although the inhibition dominates. This circumstance renders calculation of the degree of inhibition,  $n$ , inaccurate. Lindahl (150) has devised a method of avoiding this difficulty by comparing the respiration in darkness and in light strong enough to remove the inhibition by carbon monoxide. The increasing action of the monoxide is not removed by light. On the basis of the  $n$  values Lindahl calculates the values of the partition constant according to Warburg (290):

$$K = \frac{n}{1 - n} \times \frac{\text{CO}}{\text{O}_2}$$

Klein and Runnström (124) gave this equation a more general formulation and supported the introduction of a factor  $\epsilon$ , the degree of saturation (290), in cases in which the enzyme was not saturated by substrate; in this respect they disagree with Lindahl (150) and Fisher (63).

It was also claimed by Runnström (229) that the respiration of the unfertilized egg is less sensitive to cyanide than that of the fertilized egg (see also 127). Complications in the pertinent experiments have, however, been pointed out by Lindahl (152), who claims that the respiration of unfertilized eggs may also be inhibited by cyanide. This has indeed been confirmed by Robbie (221).

It was shown that the iron-containing oxidase is able to oxidize dimethyl-*p*-phenylenediamine (DPPD) at the same rate in unfertilized and fertilized eggs (231). The data were interpreted in the following way: In the unfertilized egg the oxidase is unsaturated; the comparatively low rate of oxidation in the unfertilized egg engages only a small fraction of the oxidase molecules; hence a reaction of these with carbon monoxide will have no inhibitory effect because there is still a sufficient number of oxidase molecules present. Thus the oxidase in the unfertilized egg is not blocked. The block which keeps down respiration acts in the more negative part of the chain of respiratory enzymes. When upon activation of the egg this block is removed, the oxidase becomes more saturated. Increased saturation must entail a higher degree of inhibition by carbon monoxide (205,206,229,231).

Upon addition of DPPD the oxygen consumption is raised above the value found in the newly fertilized egg. Inhibition through carbon monoxide is also considerably increased, *i.e.*, the partition constant,  $K$ , decreases. This is easily explained on the basis of increased saturation which engages an increasing number of oxidase molecules. Lindahl (150) found that inhibition through carbon monoxide increases in the course of development until nine hours after fertilization (late blastula stage). This again must be due to increasing saturation of the oxidase. The amount of this enzyme present in the uncleaved egg suffices to provide for respiration in more advanced stages also. In the period from 9–24 hours after

fertilization the partition constant,  $K$ , has a constant value although respiration increases (150). This may mean that oxidase is formed in proportion to the increase in activity of the reducing systems.

The conclusions of Runnström expressed in this way have been accepted by later workers (see 6,127). Krahle *et al.* (129) have further demonstrated the presence in broken-up eggs of an enzyme able to oxidize cytochrome *c*. This enzyme is not identical with cytochrome oxidase, but resembles it rather closely (23). The enzyme occurs in nearly equal amounts in unfertilized and fertilized eggs. It is inhibited by cyanide, azide, and carbon monoxide, but not by certain copper inhibitors. On the basis of certain results obtained by Barron (8) the authors develop an interesting hypothesis according to which an oxidase molecule linked with an inhibitor would not be altogether inactivated but would exhibit a certain activity as electron carrier. The decrease in oxidation-reduction potential of the oxidase-inhibitor compound would, however, impair its activity. This hypothesis would explain, as a metal-catalyzed oxidation, the residual respiration found in many cells in the presence of the inhibitor.

Runnström (229) and Örström (205) studied the rate of reduction of methylene blue by living eggs of the sea urchin *Paracentrotus lividus*. No difference was found between fertilized and unfertilized eggs. Ballentine (6), on the other hand, found a difference in the rate of reduction of ferricyanide and methylene blue when comparing *Arbacia punctulata* eggs in the fertilized and unfertilized stage. Ferricyanide reacts directly with reduced codehydrogenase. Ballentine thus concluded that the codehydrogenase-dehydrogenase-substrate part of the respiratory system determines the over-all rate of respiration. No attempt was made, however, to determine which of the components mentioned constitutes the limiting factor. It was found by Runnström (234) and by Korr (127) that addition of pyocyanine to unfertilized eggs may increase the rate of oxygen uptake to a value similar to that found in the fertilized egg (Fig. 10). These observations made on the same species used by Ballentine rather suggest that the coenzyme-dehydrogenase-substrate system is as active in the unfertilized as in the fertilized egg.

Lindahl (153) has recently carried out experiments which may account for the differences between the results obtained by Runnström and by Örström and those obtained by Ballentine. Lindahl compared the times that were re-

quired to reduce methylene blue by suspensions of living unfertilized and fertilized *Paracentrotus* eggs. The difference is relatively slight at 12 and 16° but increases at higher temperature (20–24°). Runnström's observations were carried out at a temperature of about 16°. Ballentine's experiments with ferricyanide acting as an electron acceptor were made at 25°, while no statement is made about the temperature in Ballentine's experiments with methylene blue. Presumably the temperature was kept at 25° in these experiments also.

Ballentine (6), like Runnström (234), proposes the hypothesis that upon fertilization a carrier is released. This carrier fills the gap existing in the respiratory system of the unfertilized egg and

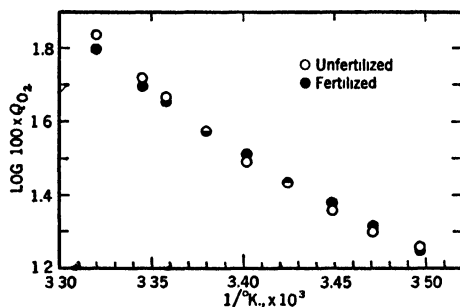


Fig. 10. Temperature characteristics of the oxygen consumption of unfertilized eggs with 0.015% pyocyanine and of fertilized eggs (127). No difference in oxygen consumption between the two kinds of eggs.

thus brings about an increase in the rate of respiration. Earlier, Runnström (229) maintained that the increase in respiration following fertilization might be due to structural changes. These would bring about contacts between components of the respiratory system which are partly separated in the unfertilized egg. Brachet (34) is inclined to give preference to the latter view. The question of the location of the gap in the respiratory system of the unfertilized egg is not yet settled and needs further experimental elucidation.

The apparent contradiction between the results with methylene blue and ferricyanide on the one hand and those obtained with pyocyanine on the other recall, however, results obtained with dry yeast. Lennerstrand and Runnström (135) found a much higher O<sub>2</sub> uptake on addition of pyocyanine than when methylene blue was added. Lindberg and Ernster (158) and Hultin (119) stated further that a homogenate of unfertilized eggs of *Psammechinus* consumed more oxygen in the presence of pyocyanine than upon addition of methylene blue. Certain substrates, such as glucose, were attacked

only when pyocyanine was present. Thus certain enzymes in the homogenate come into operation in the presence of pyocyanine, but seem to be inaccessible to methylene blue. The same apparently holds true of the intact unfertilized egg.

As mentioned above, parthenogenetic agents may be effective under anaerobic conditions also. Harvey (93), Runnström (230), and Barron (7) showed that sea urchin (*Arbacia* and *Paracentrotus*) and *Nereis* eggs might be fertilized under anaerobic conditions.

The spermatozoon enters the egg and the fertilization membrane is formed but no swelling of the sperm nucleus occurs. On transfer to normal sea water the eggs develop. These results demonstrate that respiration is not necessary to the first steps of activation. It seems of interest to inquire whether some energy-delivering processes which proceed without oxygen uptake occur in connection with the activation of the sea urchin egg. Runnström (230,233) could demonstrate with the Warburg technique that acid formation takes place upon fertilization of the eggs of *Paracentrotus lividus*. These results have been confirmed by other authors. Table V gives the quantitative data. The values are those recalculated by Örström and

TABLE V  
ACID FORMATION UPON FERTILIZATION OF SEA URCHIN EGG

Species	Acid, μmole/100 mg. N	Author
<i>Paracentrotus lividus</i>	18.3	Runnström, 233
<i>P. lividus</i>	15.0	Örström, 207
<i>Psammechinus miliaris</i>	21.1	Borei, 21
<i>P. miliaris</i>	22.9	Laser and Rothschild, 133

Lindberg (209). The acid formation is limited to the first fifteen to twenty minutes after fertilization (see also 231,232). It takes place under both aerobic and anaerobic conditions. Acid formation was also found when unfertilized eggs were placed in hypertonic or hypotonic media. The acid formation was measured both by observing the carbon dioxide evolved directly and by estimating the decrease in bound carbon dioxide occurring in the egg suspension (233). The chemical nature of the acid formed is still unknown. The data of Perlzweig and Barron (215) do not indicate any change in lactic acid content after fertilization of the *Arbacia* egg. Runnström (233; for further details see 209) compared the lactic acid

content of unfertilized eggs and eggs which had just been fertilized, i.e., eggs which were in the stage of acid formation. No difference was observed (averages for unfertilized and freshly fertilized eggs were, respectively:  $0.97 \pm 0.07$  and  $0.99 \pm 0.1$  mg. lactic acid per 100 mg. nitrogen). No changes were found to follow the fertilization with respect to the content of orthophosphate or other acid-soluble phosphate compounds (209,233,307; see also 154a).

Working with *Paracentrotus* eggs the writer found a total content of acid-soluble phosphorus amounting to 5.6 to 8.8 mg. per 100 mg. nitrogen. About 50% was orthophosphate and about 25% easily hydrolyzable phosphate ester (ATP). The remainder was difficultly hydrolyzable (154a,307). The difficultly hydrolyzable fraction was found by Lindberg (154a,156) to be constituted mainly of propane-diol phosphate, an ester which was also found present in different mammalian tissues. Örström and Lindberg (209) found a sudden breakdown of glycogen after fertilization. Calculated as glucose, the average breakdown amounted to 26.1 micromoles per 100 mg. nitrogen. The conversion of glycogen does not result in the formation of lactic acid. Some reservation must be made, however, with respect to the designation of the compound dealt with as glycogen. The reduction values found may also refer after preceding hydrolysis to polymeric carbohydrates other than glycogen. This was pointed out to the writer by Dr. Vasseur, who showed that an apparent glycogen content may be demonstrated in the jelly coat substance of sea urchin eggs even though it certainly contains no glycogen. Using the histochemical method of Hotchkiss (114), Monné (185a) has demonstrated the presence of carbohydrates within the egg which, like the jelly coat substance, are not attacked by salivary amylase.

Runnström (233) pointed out the great similarity that seems to exist between the dehydrogenase system of the sea urchin egg and the system from red corpuscles studied by Warburg and Christian (291). In the latter hexose monophosphate is oxidized under the influence of "zwischenferment" and triphosphopyridine nucleotide (TPN). Phosphogluconic acid appears as an oxidation product. A suspension of unfertilized *Paracentrotus* eggs was centrifuged, part of the sea water removed, and finally the same volume of distilled water added. The homogenates formed by stirring the egg in the hypotonic medium oxidized above all hexose mono- and hexose

diphosphate in the presence of methylene blue. The oxygen consumption in air as well as the anaerobic reduction of methylene blue was followed. Pyruvic and lactic acids were oxidized at a much lower rate, and succinic and maleic acids hardly at all.

Lindberg (156) demonstrated further that propanediol phosphate, phosphogluconic, fumaric, and malic acids strongly reduce methylene blue under anaerobic conditions in the presence of homogenates of *Echinocardium* eggs. The homogenates were prepared by freezing the egg suspension and thawing it in hypotonic sea water. Later, Lindberg and Ernster (158) measured the oxygen consumption in homogenates of unfertilized *Strongylocentrotus* eggs. The homogenates were prepared by shaking the jelly-free eggs in buffered sodium chloride and potassium chloride mixtures ( $pH$  7.7) to which certain amounts of magnesium chloride were added after disruption of the eggs. After addition of pyocyanine a rather strong oxygen uptake was observed. This was further enhanced by diphosphopyridine nucleotide (DPN). The homogenates strongly oxidized added glucose, hexose mono- and hexose diphosphate. The conversion of these substrates was not inhibited by iodoacetate. No triose phosphate was formed. The compounds in question are evidently not split according to the classical scheme of glycolysis. The homogenate system is able, however, to give energy for the phosphorylation of added adenylic acid into adenosine di- (ADP) and triphosphate (ATP). According to Hutchens *et al.* (120a) neither production nor consumption of lactic acid appears to be of quantitative importance as energy-yielding processes for *Arbacia* eggs during their first 24 hours of development. Furthermore they were not able to find any triose phosphate dehydrogenase activity in the *Arbacia* egg.

Hultin (119) using a technique similar to that of Lindberg (158) homogenized eggs of *Psammechinus* in a calcium-free medium. Pyocyanine was added to the homogenate adjusted to  $pH$  7.0. As shown in Figure 11, oxygen consumption took place in this system. On addition of calcium chloride an approximately threefold increase in the rate of oxygen consumption occurred. In the course of the experiment, however, the rate decreased under that prevailing in the calcium-free medium. Full calcium effect was obtained with concentrations of added calcium chloride of 0.003 *M*. Analogous results were obtained when calcium was added to homogenates containing such substrates as malic acid, which enhances respira-

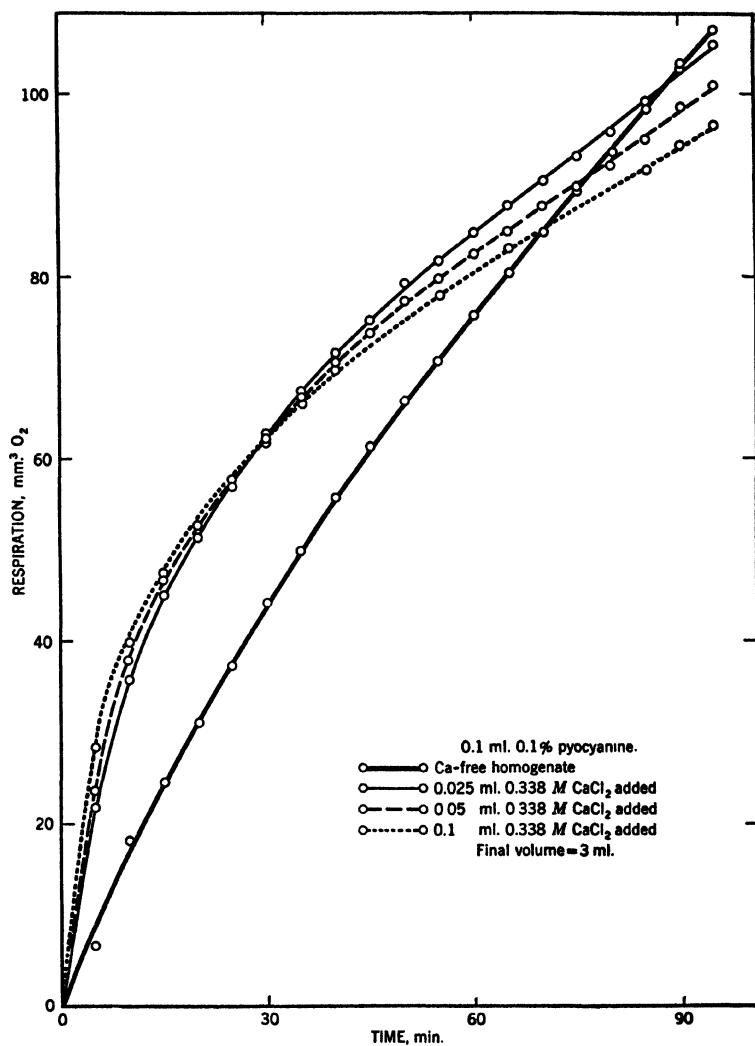


Fig. 11. Effect of calcium added to calcium-free homogenate on the oxygen consumption of *Psammechinus* eggs (119).



tion in the calcium-free medium. Under anaerobic conditions addition of calcium incited strong acid formation as measured by the liberation of bound carbon dioxide. Acid formation also takes place under aerobic conditions but could not be accurately estimated. The calcium effect described by Hultin is of great interest in view of the important role calcium no doubt plays in the activation of the egg, as first emphasized by Dalcq (51), Heilbrum (103), and Pasteels (212) (see Sect. VI). Hultin's data furnish evidence to the effect that calcium may activate both respiration and anaerobic splitting processes in the egg. According to Hultin addition of sodium chloride to the egg suspension in a calcium-free medium to increase the osmotic concentration 50–100% enhances the oxygen uptake in the presence of pyocyanine two- to three-fold. A decline of the rate also soon takes place under these conditions. Addition of trypsin (commercial preparation) also gave an increase of oxidation under certain conditions; above all acid formation is strongly enhanced upon the addition of trypsin.

Runnström (233) demonstrated the presence of a strong ATPase in the unfertilized egg of *Paracentrotus lividus*. According to Connors and Scheer (44) an increase in the ATPase activity probably occurs after fertilization. They found that homogenates from fertilized eggs of *Strongylocentrotus purpuratus* hydrolyze 10.28  $\mu$ g. phosphorus per hour and milligram nitrogen, whereas the corresponding value for homogenate from unfertilized eggs amounts to 4.13. Runnström suggested long ago (233, p. 262) that the constancy of the value of hydrolyzable, acid-soluble phosphorus before and after fertilization is only apparent. A breakdown of ATP may be compensated by synthesis which takes place at the expense of energy released in the processes observed as an acid formation. It follows from the report given in Sects. II and V that ATP exerts an effect on the egg. The evidence, although still incomplete, tends to show that ATP possibly plays a role in bringing about the structural changes accompanying the activation of the egg.

Lindberg (157) determined the turnover of ATP in the unfertilized and fertilized *Psammechinus* egg. Orthophosphate containing radioactive phosphorus was added to the sea water. Samples were taken at intervals and the specific activity of the ATP estimated. As evident from Figure 12, an equilibrium was very soon attained in the unfertilized egg. This must mean that only a small part of

the phosphate content of the egg takes part in the reaction. The participating phosphate must have its site outside the permeability barrier that prevents the penetration of orthophosphate into the interior of the egg. In the fertilized egg the specific activity of ATP

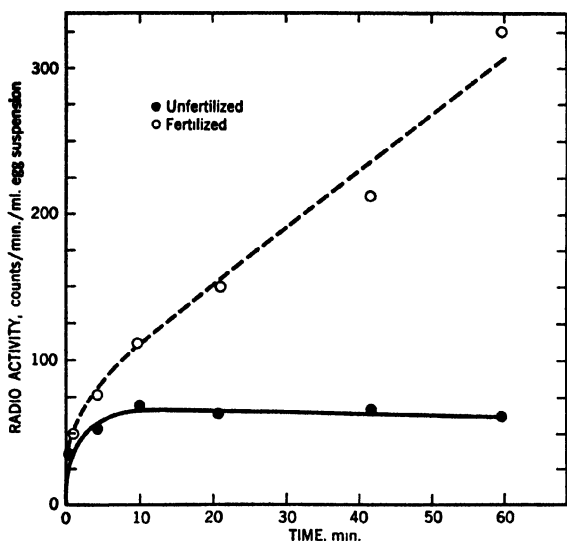


Fig. 12. Turnover of pyrophosphate phosphorus in adenosine triphosphate in unfertilized and fertilized eggs (157).

increases during the whole experiment. Here the exchange takes place between the exterior and the whole quantity of orthophosphate within the egg. Abelson (1) showed earlier that permeability to phosphate increases forty fold after fertilization. The new level of permeability is attained seven to ten minutes after fertilization. Lindberg's data show that a rather lively turnover of ATP is displayed in the surface of the unfertilized egg. ATP may play a role in maintaining a certain state in the surface necessary to assure normal cortical reaction upon attachment of the sperm. This contention receives support from the data referred to previously concerning the influence of ATP on the capacity for formation of a normal fertilization membrane in *Arbacia* eggs (see Sect. II).

Runnström (233) demonstrated the presence of DPN in the *Paracentrotus* egg. No difference in the content of this coenzyme was found when extracts from fertilized and unfertilized eggs were compared. Jandorf and Krahle (121b) estimated the DPN content of *Arbacia* eggs at 250–500  $\mu\text{g.}$  per gram wet weight, of which 25–40% is in an alkali-stable form. The same group of workers (129) also found 2–3  $\mu\text{g.}$  diphosphothiamine per gram wet weight of *Arbacia* eggs. Barron and Goldinger (9) have made similar statements.

The studies carried out by Örström (208) on the eggs of *Paracentrotus lividus* indicate that an increased production of ammonia is connected with fertilization. This ammonia production subsides very soon, in about ten minutes. From experiments with egg homogenates, Örström infers that the ammonia is produced mainly by deamination of adenine bound to macromolecules, probably nucleic acid. Furthermore, Örström contends that fertilized eggs can form glutamine from added ammonia and glutamic acid. Unfertilized eggs, on the other hand, are unable to synthesize glutamine; they can only break it down into the components glutamic acid and ammonia. These results if substantiated are very important and may indicate that fertilization brings about a change from a mainly catabolic type of metabolism to a type in which anabolic processes take place at the expense of catabolic processes. Hutchens *et al.* (120a) also found ammonia production in *Arbacia* eggs, but they did not pay special attention to the period immediately following fertilization.

### VIII. Attempt at a Comprehensive View. Enzyme Activation as a Principal Factor in the Incitement of Development

The role of enzymes in the activation process has naturally often been in the minds of workers on this subject. Runnström (237,240) recently emphasized the hypothesis that the incitement of development may be due to activation of enzymes. Undoubtedly many of the data referred to above point to this conclusion. The activity of enzymes is controlled within the organism by inhibitors and activators. Study of the regulation of enzyme activity promises to develop in a manner which has great bearing on cell physiology, see (46,201,219).

Loeb (165) assumed that release of a catalyst followed the breakdown of a calcium lipid compound in the cortex. This release rendered the catalyst capable of reacting with its substrate. Lillie (139, p. 27) discussed the possibility that fertilizin operated either by activating some substance in the spermatozoon or by being transformed from an inactive to an active state by some substance in the spermatozoon. Furthermore, Lillie suggested that fertilizin might be enzymelike in nature.

Lillie apparently established that fertilizin, the sperm-agglutinating substance, is necessary to fertilization. This, however, can nowise be regarded as definitely proved. The cessation of fertilizin production in artificially activated *Arbacia* eggs and the simultaneous cessation of fertilizability seemed to lend strong support to Lillie's contention (see 196). Tyler (277, p. 193) points out that the jelly coat is removed by the butyric acid treatment applied in the latter experiments. Since fertilizin corresponds to the jelly coat substance, no fertilizin activity can possibly be displayed (for further discussion see 277).

Knowledge of the chemical nature of the jelly coat substance suggested that it might function as an inhibitor. Its great potency in inhibiting blood clotting was given in Sect. III. Heparin and like substances in fact exert an inhibitory action on several enzymic processes. Runnström (241) dissolved dry jelly coat substance, prepared as mentioned above, in sea water. The solutions quite clearly produced inhibition of the membrane dissolution which normally occurs in the blastula stage. The embryos were often found still within the membranes in the gastrula stage or they escaped as blastulas, but the membranes were present on the bottom of the bowl. In the control with approximately the same number of eggs per milliliter the membranes dissolved. This preliminary experiment carried out on *Arbacia lixula* points to the conclusion that jelly coat substance inhibits the proteolytic enzyme acting in the blastula stage on the membrane.

The following observation also indicates inhibitory action exerted by jelly coat substance. Unfertilized eggs of *Arbacia lixula* were exposed to 0.02/0.1% solutions of jelly coat substance (241). When the eggs were inseminated, only the gelatinous rim was formed, even when the control eggs in pure sea water formed a perfect membrane. Evidence presented above (Sect. V) showed that a coagulation

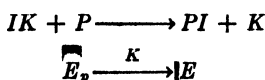
process is involved in the final step of membrane formation. When the jelly coat substance is present this final step is probably prevented by an inhibitory action on the coagulation process. In these experiments the fertilizing power of the spermatozoa was not inhibited by the jelly coat substance. Even after this substance had been removed by centrifuging of the sperm suspension, the spermatozoa were able to activate eggs, with subsequent normal membrane formation.

The jelly coat substance has an activating effect on the sperm (see Sect. IV). Tyler (272) showed, however, that rather strong egg water of *Strongylocentrotus purpuratus* considerably lowered the fertilizing power of a sperm suspension of this species. This result at least does not conflict with the idea that the jelly coat substance exerts an inhibitory action on enzymes.

If a modified fertilizin theory is to be defended, the assumption must be made that glycoproteins similar to the jelly coat substance are also present in the inner layers of the egg, at least in the vitelline membrane and also in the cortical layer. The vitelline membrane is the seat of a specificity which apparently is equal to that of the fertilizin. Furthermore, the naked eggs are agglutinated just like the jelly coats by the action of the antifertilizin of sperm and of other basic proteins (clupein, salmin). According to Tyler (277, p. 191) this means that a layer of fertilizin remains intimately bound to the surface. Certainly it means that the vitelline membrane has an acid character, and this may be due to the presence of acid glycoproteins. According to Monné (185a), carbohydrates are present in the hyaline layer (see also Section VII). This layer is, however, exuded from the cortex subsequent to fertilization. In the unfertilized egg the material of the hyaline layer thus forms part of the cortex.

Runnström (231) contended that an inhibitor of proteolytic enzymes was present in the vitelline membrane and the cortical layer. (This hypothesis is a modernized reformulation of the inhibition theory of Lillie, 137, and of Bataillon, 12; Lillie even points out the jelly as inhibitor in *Nereis* eggs.) The inhibitor may be identical with a heparinlike substance. The inhibitor is assumed to be removed, however, by the reaction with substances present in the sperm. The enzyme, or rather a kinase acting on a proenzyme

(see 23), may then be released. The reactions may be formulated in the following way:



where  $I$  is the inhibitor,  $K$  a kinase,  $E_p$  the proenzyme or enzymogen, and  $P$  a protein combining with the inhibitor.  $P$  may represent a specific component of the surface of the spermatozoon,  $P^1$ , or a more unspecific basic protein,  $P^2$ , released from the nucleus of the attached spermatozoon. It may be significant in this connection that the basic sperm antifertilizin is able to induce activation of the egg (see Sect. VI). The reaction expressed in the equation may release the antifertilizin of the egg,  $P^3$ , which may further contribute to the release of enzyme.

The proposed scheme is a modified fertilizin theory in which, however, an inhibitory action is assigned to the "fertilizin," but the tentative scheme submitted above need not necessarily be connected with the fertilizin theory. Nevertheless analogies between the behavior of the jelly coat and the cortical layers suggest that the reaction between the fertilizin and the sperm receptor displayed outside the cytoplasm may serve at least as a model of what happens inside the cytoplasm.

The nature of the initiating reaction can of course not be indicated at present. The writer finds it reasonable to assume that proteins are involved in basic changes occurring upon activation of the egg. Lundblad (167) and Runnström (241) extracted lyophilized unfertilized and fertilized eggs with a dilute salt solution. Gelatin-liquefying activity was found in extracts from eggs frozen three minutes after insemination. Only very weak activity was present in the extracts from unfertilized eggs (see Fig. 13). When these were kept under sterile conditions the activity increased, apparently following a linear function. The enzymic activity evidently disappeared soon after fertilization. No activity was demonstrable in extracts from eggs frozen thirty to forty minutes after fertilization and no spontaneous activation seemed to occur. These preliminary results indicate that one or several proteolytic enzymes are activated immediately after fertilization. It was found that the proteolytic activity follows the components 3 and 6 in Monroy's (190a) electrophoretic diagrams (see Sect. V).

The writer is inclined to assume that the proteolytic enzyme brings about certain changes in the proteins which result in unmasking reactive groups. An increased reactivity of the colloids mainly the proteins, subsequent to fertilization, is indicated by the increased binding of cephalin and of carbohydrate to protein (see Sect. V). According to Örström (208) added ammonia accumulates many times more in fertilized than in unfertilized eggs. Loeb (162) observed that both unfertilized and fertilized *Strongylocentrotus* eggs may be stained with neutral red. When returned to pure sea

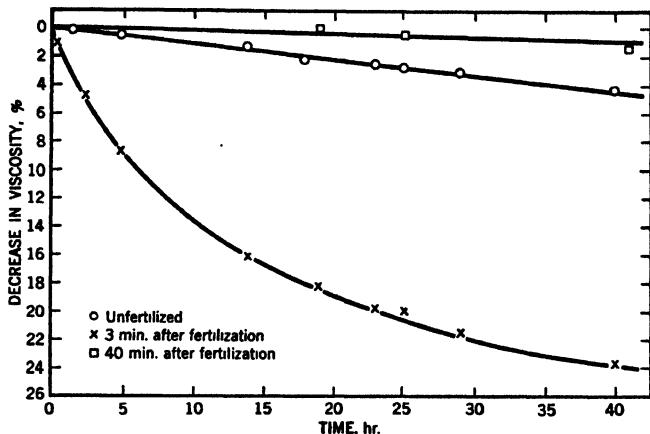


Fig. 13. Decrease in viscosity of gelatin under the action of egg extracts from unfertilized eggs (167). The viscosity of nontreated gelatin coincides with the uppermost curve.

water the unfertilized eggs gradually lose their color. Conversely, the fertilized eggs retain their color, which merely deepens with time. Runnström (227) confirmed these findings with the stain brilliant cresyl blue and *Paracentrotus* eggs. Gellhorn (67) and Runnström (227) found that uptake of the dye ceases at a higher pH in the unfertilized than the fertilized eggs.

The increased reactivity may also result in the gelation or coagulation which occurs upon fertilization. Positive and negative unmasked groups may attract each other, thus connecting certain proteins to a gel. These connections may also be responsible for the higher

stability of the cytoplasm of the fertilized as compared to the unfertilized eggs (see Sect. V).

Finally, certain unmasked groups may exert enzymic activity. This may explain the simultaneous activation of several enzymes in connection with activation: a carbohydrate-splitting enzyme of unknown nature (209), ATPase (44), and alkaline phosphatase (299). A splitting of phosphatides in the protoplasmic surface is also probable if our assumption of the polyspermy-preventing mechanism holds true (see Sect. IV). Observations with darkfield illumination also speak in favor of changes in the lipides (see 224,226, and Sect. V). A certain lag is to be expected between the primary changes in the proteins and such subsequent changes as gelation, etc.

Heilbrunn and his school (103) assume that a release of calcium plays a decisive role in the process of activation. Part of the evidence has already been reviewed above. The data presented by Mazia (175) indicate an appreciable release of calcium upon fertilization of the *Arbacia* egg. Örström and Örström (210) and Monroy-Oddo (195) have also presented data to the same effect. It would be of great value to study the question of calcium release with some method independent of those used by the workers mentioned.

Heilbrunn (101) was the first to present evidence favoring the coagulation theory of activation when he demonstrated the increased viscosity following fertilization (see Sect. V). In further work he was impressed by the similarity between the surface precipitation reaction which occurs after the injury of the cell surface and blood clotting (102). In both processes calcium is a necessary factor. Heilbrunn's contention is that calcium operates in a kind of clotting process which occurs in the surface precipitation as well as in the activation.

The analogy between blood clotting and the changes occurring upon activation of the egg are no doubt very suggestive. The writer believes that the blood clotting process gives a useful model of a complicated course of enzyme activation which may recall what happens in the incitement of development. From other fields of enzyme chemistry as well, analogies to the scheme proposed above present themselves. The activation of trypsinogen to trypsin is an autocatalytic reaction which, however, may also be enhanced by



the enzyme enterokinase. Of special interest is the enhancing influence of calcium on the activation of trypsinogen (see 201).

Connors and Scheer (44) showed that calcium activates the ATPase in the sea urchin egg. It seems evident that ATP and ATPase play a role in fertilization. It has been reported above that ATP is important in maintenance of the fertilizable state of the eggs. Furthermore, ATP is one of the factors which effect the changes in the colloids occurring subsequent to activation (see Sects. II and V).

Hultin's (119) results (see Sect. VII) indicate that calcium has considerable capacity for activating enzyme processes involved in acid formation and respiration. It also influences the state of the colloids in the homogenate. It is possible that the effects described by Hultin are indirectly caused by an influence of calcium on ATPase.

In this connection the enzyme precursor "plasminogen" may be recalled. It is present in mammalian blood plasma and may be activated to a proteolytic enzyme plasmin, under the influence of extracts from tissues and certain bacteria (4), but coarser methods also such as treatment with chloroform can be used (42). This is closely analogous to activation of the egg, which may be brought about by the action of specific complicated substances as in fertilization. In artificial parthenogenesis, however, relatively simple organic compounds may also operate. Chloroform has, in fact, been used as an activating agent in experiments both with sea urchin and frog eggs (see 16, and Sect. VI).

The most essential effect of activation is no doubt the increased rate of certain anabolic processes. One of these is the synthesis of thymonucleic acid demonstrated by Brachet (30) (see also 33,34). It must be assumed, however, that certain anabolic processes also occur in the cytoplasm. The division of the central bodies and the concurring cell cleavage has been regarded above as an indicator of anabolic processes leading to the self-propagation of organized cytoplasmic units. This process was considered the most exacting of those occurring after activation of the egg. The decline of the female central body may be regarded as the effect of the decline of anabolic processes in the egg after the conclusion of the meiotic divisions (see Sect. VI).

The revival of anabolic processes after fertilization may be due both to the activation of certain enzymes and to the formation of structures which may serve as templates for the synthesis of high molecular compounds. The synthetic processes during the early phase of development from fertilization to the early gastrula stage result probably only in changed quantitative relations between different compounds present in the egg. From the beginning gastrula stage on, new compounds arise. In this stage antigens appear, which are not demonstrable in the earlier stages (86, see also 45).

It is not possible as yet to indicate the sequence of the different processes released in the activation of the egg. Interactions between later and earlier links in the concatenated initiating reactions probably occur. This may contribute to give the over-all process a certain autocatalytic character. If, for example, the process *A* causes the process *B*, and this in its turn *C*, the last process—let it be for example, release of calcium—may accelerate process *A*, which again will accelerate *B* and *C*. Parthenogenetic agents may attack different links in the chain of events but in view of the interaction the whole chain might come into operation irrespective of which link was primarily affected. This allows for a variety of parthenogenetic agents.

Certain of the concatenated processes may occur spontaneously. This is the basis of cytoplasmic maturation described in Sect. II. A proenzyme may, for example, gradually be transformed into the active enzyme but only the release of a kinase gives a concentration sufficient to release the over-all process. The presence of subliminal concentrations, however, brings about a more advanced state of maturation.

In his earlier work Lillie (142) particularly stressed the similarity between activation of the egg and stimulation processes in general. An essential role was ascribed to the protoplasmic membrane and changes in its permeability and its state of polarization.

As yet no one has been very successful in applying the methods of electrophysiology to the problem of activation (see Rothschild, 222, and references therein). If these methods could be applied, new possibilities would be opened of following the initiating, rather rapid processes which provide protection against polyspermy and bring about reorganization of the egg surface.

Heilbrunn's theory of activation is only part of his general theory of cell stimulation, according to which stimulation involves intracellular clotting.

Interesting analogies no doubt present themselves between the activation of the egg and stimulation of nervous tissues. One may, for example, point out the general similarity between cytoplasmic maturation and the building up of an "excitatory state" in the central nervous system. In the egg, as in the neuron separate stimuli may contribute to the attainment of a threshold level which releases a response.

Surprising similarities prevail between the changes following the fertilization of sea urchin eggs with coarsened structure, and the "chromatolysis" occurring in the regenerative cycle of motoneurons (19). These analogies were examined in a more detailed way by Monné (185).

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# METABOLISM OF SEMEN

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## I. Introduction

Early investigators of semen were in the main concerned with the elaboration of structural details of the spermatozoa; but there were also some studies of a more physiological and chemical nature such as those initiated by Miescher (209) on the nucleoprotamines in fish spermatozoa, and later continued by several distinguished investigators (32,137,161,204,237,243,267,283,284). More recently, however, the expanding practice of artificial insemination as a means of animal breeding provided a fresh incentive to physiological and biochemical research on semen; equally stimulating were clinical

studies on infertility, which showed that, in many aspects of human and animal reproduction, the assessment of the physiological status of semen is of prime diagnostic value. There is a considerable literature pertaining to this subject, which has been reviewed on numerous occasions (1,26,27,33,61,78,86,90,101,121,179,211,225,229,242,312,318,320,322). A perusal of these monographs and articles reveals the acute need for the development of truly scientific criteria of male fertility and the demand for improvement in the standards of sperm evaluation which offer guidance in the diagnosis of sub-fertility and sterility.

At present the methods available for the appraisal of "semen quality," apart from the fertility test based upon the actual fertilization of the ovum, fall into three main categories. One important class consists of methods based on *microscopic* examination of semen, involving such procedures as determination of sperm density, percentage motility, viability, differential count of abnormal forms, differential staining for live and dead cells, ability of sperm to penetrate the interface between semen and cervical mucus, behavior in artificial media, resistance to dilution and to the action of extraneous agents, bacteriological examination, etc. The second category involves measurements of electrical changes which are associated with the movements of spermatozoa (253). The third category comprises the *biochemical* methods in which the physiological condition of the semen is assessed by measuring the rate of metabolic processes which take place in semen; the principle underlying these methods being that the two chief metabolic processes of semen, namely, respiration and glycolysis, are both a direct outcome of the metabolic activity of the sperm cells, and that the rate of both the respiration and the glycolysis is determined in the first place by the number of spermatozoa in the semen. This point needs to be emphasized, since "whole semen," i.e., as ejaculated, is made up not of spermatozoa alone but contains apart from the cells a fluid, the so-called "seminal plasma," which is composed of secretions contributed by the various accessory organs of reproduction. The composition of semen varies according to the relative contribution of the accessory organs and this explains the frequent changes in semen observed even in the same individual. Unfortunately these circumstances have not always received necessary recognition and

many data, especially the earlier ones, concerning the chemical composition and the metabolic activity of semen were obtained with whole semen, with no clear distinction drawn between the sperm and the seminal plasma and without sufficient attention to the fact that the ratio of sperm to plasma in whole semen is by no means constant but is subject to considerable fluctuations. Yet this precisely is the factor which must be taken into account in all biochemical investigations of semen. It explains, for instance, why the analysis of whole semen, even if restricted to the same experimental subject, and even if carried out under identical experimental conditions, need not always yield the same quantitative results as would, for example, the analysis of blood in the same individual.

Quite apart, however, from the practical value derived from the examination of semen, biochemical research on semen and, more particularly, the study of the metabolism of semen, offers the investigator some unique opportunities such as are not available to him in the metabolic investigation of any other tissue or body fluid. What makes semen a unique experimental object is that it provides a rare instance of an animal tissue composed of but one type of cell, the spermatozoon, so that all metabolic measurements can be related specifically to the sperm cells, and the metabolic activity observed in semen can be expressed directly in terms of cell number without the application of less satisfactory criteria such as dry weight of tissue, nitrogen content, or any of the other commonly used indices. Of equal importance is the fact that in the semen the spermatozoa are completely detached from each other, each leading a life of its own as it were, yet none capable of undergoing a process of cellular division or multiplication, so that in effect the semen can be regarded as a uniform cell suspension with strictly defined cell density. Furthermore, the spermatozoa are endowed by nature with two highly specialized biological functions, fertility and motility, which not only distinguish them sharply from other cells, but which at the same time set at the disposal of the investigator two clearly defined and specific biological criteria of sperm activity to be used in conjunction with his observations on the metabolic activity. Another unusual aspect is the capability of semen to survive for a long time under certain conditions of storage *in vitro*. Again, apart from the practical bearing on problems

of fertility and artificial insemination, the fact, for instance, that mammalian spermatozoa can be preserved *in vitro* for many days in satisfactory condition, retaining their motility and fertility, makes it possible to study the metabolism of these cells *in vitro* for extended periods of time and in complete separation from the rest of the animal body.

This discourse on the unusual features of semen as distinct from other tissues would not be complete without a reference to the unique state of nutritive requirements of the spermatozoa. Some thirty years ago, when studies on the metabolism of semen were still almost exclusively restricted to fish and sea urchin spermatozoa, Lillie, in his now classical book on *Problems of Fertilization* (163), summed up the then prevailing views by pointing out that:

"Spermatozoa are probably incapable of receiving nourishment outside of the gonad after they are fully differentiated; certainly in the case of all forms with external insemination there is no opportunity for the restitution of substance. We must therefore regard these cells as charged with their full available store of energy in the testes and their capacity for locomotion as thus determined and limited. They therefore have a strictly limited period of life, the duration of which will be determined by their activity. The store of energy is saved when they are motionless and expended when in motion. Thus we find that sperm suspensions will retain their fertilizing power for a relatively long time if activity is reduced, and will lose it rapidly if activity is great."

The statement contained in the last two sentences could hardly be questioned today and, if anything, the experiences derived from studies on mammalian semen give additional strength to the view that the best way to prolong the life span of sperm, at least under storage conditions *in vitro*, is to reduce temporarily the motility and metabolism of spermatozoa by artificial means such as, for instance, the lowering of temperature. On the other hand, however, chiefly as a result of the work on mammalian semen, it is no longer possible to regard the intracellular nutrient reserve of spermatozoa as the decisive factor in the sperm nutrition. The studies on carbohydrate metabolism, and, more specifically, the researches concerning the occurrence and function of seminal fructose (see Sect. IV) have proved that mammalian spermatozoa, at any rate, are to a large extent independent of the small nutrient reserve which they acquire during their generation in the testes, and that they are

capable of extensive "feeding" on fructose which they assimilate from the seminal plasma and metabolize readily, thereby obtaining life energy. This behavior of spermatozoa further adds to their uniqueness as an object for metabolic studies; indeed so far as their nutrition is concerned they resemble more a culture of microorganisms in a nutrient medium rather than the other animal tissues with their complicated supply of nutrient material via the blood capillaries.

Mention should be made of yet another circumstance which determines to a considerable extent not only the nutritive requirements but also the function of the enzymes engaged in sperm metabolism, namely, the remarkable permeability of the sperm cell. It explains the high velocity with which exchange reactions take place between the spermatozoa and the surrounding medium, whether this be the seminal plasma or an artificial storage medium. It probably provides also the explanation for our observations that a number of intermediary enzymes, particularly those concerned with fructose metabolism of spermatozoa, can be demonstrated and examined in live sperm directly, without cell disintegration, which is the usual prerequisite to the study of intermediary enzymes in other animal tissues. Even more remarkable is the ease with which large molecules, *e.g.*, enzymes such as, for instance, hyaluronidase, can detach themselves from the sperm structure to be released by the spermatozoa into the surrounding medium. Such phenomena are thought to be involved in the actual performance of the fertilizing function of spermatozoa toward the ovum.

One of the peculiar features of mammalian spermatozoa is their sensitivity to the so-called "temperature shock," which occurs when the freshly collected semen is suddenly brought from body temperature to 5–10°C. It leads to irreversible lowering of motility and decrease in metabolic activity (42,54). This is in contradistinction to the fact that sperm cells are capable of survival in semen frozen very rapidly in liquid air, and subsequently thawed quickly by plunging into warm medium (99,120,170,226,275,278). Another characteristic feature is the behavior of spermatozoa toward changes of pH. In the case of rabbit semen, for example, which normally has a pH of 7–8, spermatozoa have been shown to retain partial motility at pH values as low as 5 and as high as 8.8. However, full



motility was seldom recorded outside the range 6.2 to 7.9 (44). Even spermatozoa rendered immotile by lowering the pH to 5 can still be revived by bringing back the pH to normal provided, however, that they have not been exposed too long to the adverse pH (57). Equally significant is the dilution factor; unless the composition of the diluent is carefully adjusted to suit all requirements of the sperm cells, dilution of semen can cause serious damage and a marked decrease in the metabolic rate of spermatozoa. This motivated the extensive search for improved "artificial media" in the practice of artificial insemination (39,77,135,230,260,261,321). In recent times increasing attention is being devoted also to the effect on spermatozoa of a number of extraneous factors such as irradiation (56,98,136,155), atmospheric pressure (70,315), and bacterial contamination (65,79,131,134,263).

## II. Considerations on the Composition of Semen

Of the two components of whole semen the spermatozoa are generated in the testes, while the other, the seminal plasma, originates in a number of accessory organs of reproduction, which in man and higher mammals include the epididymis, vas deferens, ampulla, the seminal vesicle or an analogous organ, prostate, Cowper's gland, and the various urethral glands. The function of the testes as well as that of the accessory glands of reproduction is subject to strict and intricate endocrine control by various hormones, particularly those produced in the testes and the anterior pituitary.

### A. STRUCTURAL AND FUNCTIONAL CHARACTERISTICS OF SPERMATOZOA

The peculiar features which distinguish the metabolism of semen from that of other animal tissues can be traced to a single cause, namely, the morphological structure of the sperm cell. In the vast majority of animal species capable of sexual reproduction the spermatozoa assume the form of slender, linear cells, either filiform throughout or enlarged at one end. In many species it is possible to distinguish clearly in each mature spermatozoon three regions known as the head, midpiece, and tail. However, the actual variety in form is enormous, and ever since Leeuwenhoek's microscopic observations

on the "seminal animalcules" (156) all observers laid stress on the fact that, in contradistinction to the egg cell, the sperm cell is hardly alike in structure in any two, even closely related, species; in fact, at one time two distinguished authors, Wagner and Leuckart (306) were able to remark that it is quite feasible to infer accurately the systemic position and name of an investigated animal from the mere microscopic appearance of the spermatozoa. This statement has lost none of its significance since then.

Considerable progress has been made in morphological studies on semen and new structural details have been discovered in spermatozoa with the help of more delicate procedures, such as the cytochemical methods (see 203) or the application of the electron microscope (14,91,269,270,274). Particularly instructive are the electron micrographs of bull spermatozoa obtained recently with the use of the Delft electron microscope by Bretschneider and Wouterra van Iterson (31); these photographs reveal clearly a number of structural details such as the fraying of the broken end of the sperm tail into the subfibrils of the axial filament and the spiral fibrils of the cortical sheath which surrounds the axial elements. No doubt both systems, the axial filament and the cortical helix, are fulfilling a role of some importance in controlling the movements of the sperm tail, but precisely how it is done remains unknown at present. Equally scarce is our present knowledge concerning the chemical nature of the contractile substance of the sperm tail. A certain similarity in function to muscular contraction has prompted several authors to ascribe to the contractile substance of the spermatozoan tail a nature similar to muscle myosin and even a function corresponding to that of myosin adenosinetriphosphatase (60). However, such claims are not acceptable for the time being since they were not accompanied by proof that the spermatozoa, before used for the experiment, were completely freed from other seminal phosphatases, which act on a number of phosphoric acid esters including adenosine triphosphate (see Sect. III. C.). Another matter of speculation at present is the significance of the tender protoplasmic cap, the so-called "head cup" or "galea capitis," which has been seen in many species to cover the apical part of the sperm head and to which attention has again been drawn more recently by Blom (23). Another phenomenon, the physiological role of which still escapes us, is the appearance of the so-called "kinoplasmic

droplet" attached to the spermatozoan midpiece (16,153,205,248). These kinoplasmic droplets do not appear as a rule, at least not in any great number, in the ejaculated semen but in spermatozoa removed from the testis or epididymis they may be seen quite frequently, undergoing a curious process of migration. If semen from the caput epididymis of a mouse, for example, is examined under the microscope, the kinoplasmic droplets are found to be attached to the neck or anterior end of the midpiece, but by the time the spermatozoa have reached the cauda epididymis and approached the vas deferens, the droplets have already taken up position at the posterior end of the midpiece. While some authors assume that the droplet is merely a remnant of spermatid protoplasm with no special significance, others consider that it serves to nourish the spermatozoa during their passage through the epididymis and still others regard it as being of great importance for sperm locomotion. Whatever the true function of the kinoplasmic droplet may be, doubtless its presence is an indication of incomplete maturation and is connected with the important "ripening process" which spermatozoa undergo in the epididymis (205,223,329). As will be shown later, the metabolism of spermatozoa also undergoes some characteristic transformations during the "ripening process." The possibility of some link between these changes and the function of the kinoplasmic droplet awaits further exploration. Indeed, the participation of the epididymis itself, both in the nutrition as well as in the metabolism of spermatozoa, is far from clear, apart from the long-established and most essential role which the epididymis fulfills in acting as a store organ for spermatozoa.

Since both the respiration and glycolysis of semen represent functions of the spermatozoa themselves, knowledge concerning the sperm density in semen is of great importance in every metabolic study. Whereas in some animal species, especially in some lower ones, the emitted semen takes the form of a very dense and thick suspension of spermatozoa firmly packed together and with hardly any seminal plasma to speak of, in other species, notably among the higher animals, the sperm cells in semen are in a much less concentrated state owing to the large proportion of seminal plasma. However, even among the higher animals the degree of dilution of the sperm with seminal plasma varies enormously as can be seen

from Table I. A single ejaculate of ram semen, for instance, amounts only to 0.7 to 2 ml. but is distinguished by a very high concentration of spermatozoa, 2-5 million per microliter semen; on sharp centrifugation it separates more or less like whole blood, into two approximately equal volumes of cells and plasma. On the other hand, boar semen, although very voluminous (150-500 ml. in a single ejaculate), has but a small proportion of spermatozoa, 25-300 thousand per microliter semen, and its large bulk is made up for the most part by the seminal plasma. Naturally, the ram semen, because of its high concentration of spermatozoa, is a particularly suitable object for metabolic studies on spermatozoa. Boar semen, on the other hand, with its high proportion of fluid, lends itself rather to chemical studies on the seminal plasma. Human semen, it may be noted, has a much lower sperm density than ram or bull semen. This largely explains the failure of those investigators who employed human semen in their search for intracellular components to detect the cytochromes or the phosphorylating enzymes in spermatozoa. When, however, ram and bull semen were used instead, it was possible to demonstrate without difficulty the existence in mammalian spermatozoa of all cytochrome components and the phosphorylating enzymes of glycolysis (187-189).

TABLE I  
SPECIES DIFFERENCES IN COMPOSITION OF SEMEN

Species	Volume of single ejaculate		Sperm density in semen	
	Normal variations, ml.	Most common value, ml.	Normal variations, sperm/ $\mu$ l.	Average value, sperm/ $\mu$ l.
Boar	150-500	250	25,000-300,000	100,000
Bull	2-10	4	300,000-2,000,000	1,000,000
Cock	0.2-1.5	0.8	50,000-6,000,000	3,500,000
Dog	2-15	6	70,000-900,000	200,000
Fox	0.2-4	1.5	30,000-250,000	70,000
Man	2-6	3.5	50,000-150,000	100,000
Rabbit	0.4-6	1	100,000-2,000,000	700,000
Ram	0.7-2	1	2,000,000-5,000,000	3,000,000
Stallion	30-300	70	30,000-800,000	120,000
Turkey	0.2-0.8	0.3		7,000,000

The chief obstacle in studies of the chemical composition of spermatozoa has been the lack of sufficient quantities of material

for analysis as well as the considerable difficulty encountered in attempts to separate the spermatozoa from semen or to wash the cells completely free from seminal plasma without at the same time damaging them seriously. Even more awkward is the separation of the sperm head from the sperm tail as a preliminary to an independent analysis of the two parts of the sperm cell. Miescher (209), who was the first to consider this problem, selected for his studies fish spermatozoa, where the separation can be accomplished relatively easily. He chose salmon spermatozoa, in which a treatment with water or dilute organic acids suffices to remove completely the tail and midpiece, thus making it possible to obtain a suspension of "pure" sperm heads. A similar procedure, however, in the case of mammalian spermatozoa would be of no avail; their disintegration requires much more powerful devices such as the use of an ultra-sonic vibrator or a rapid mechanical shaker. But even with "pure" sperm heads of fish, as prepared by the supposedly mild processes, it is by no means certain whether or not the treatment causes some loss in the head cytoplasm. Consequently, all analytical results obtained with such material must be considered with caution and reservations.

#### B. SPERM NUCLEIC ACID

In discussing the problem of sperm nucleic acid, account must be taken first of all of the characteristic change from ribonucleic to deoxyribonucleic acid, which occurs during spermatogenesis, *i.e.*, in the course of the stepwise transformation which begins with spermatogonia and leads through the stages of primary spermatocytes, secondary spermatocytes, and spermatids to the formation of mature spermatozoa. Evidence which supports this change in nucleic acid is derived mainly from spectrophotometric studies (36) and cytochemical investigations carried out with the help of nucleases (29,30).

In mature spermatozoa of salmon, herring, and trout, the nuclear component of the sperm head is a nucleoprotamine composed of two parts—the nucleic acid, which is of the desoxyribose type (237), and the basic protamine. A recent analysis of amino acids in salmine sulfate by Tristram (298) suggests a molecular weight of 8000 and a peptide chain of 58 residues: 40 arginine, 1 isoleucine, 2 valine,

4 proline, 3 glycine, 1 alanine, and 7 serine; the protamine sulfate contains 19.85% sulfuric acid (40 equivalents per molecule), sufficient to combine with all the arginine residues in the molecule. Pollister and Mirsky (237) have extracted the nucleoprotamine from trout sperm with 1 *M* sodium chloride and obtained a characteristically fibrous product which accounts for 81.5% of the dry lipide-free mass of spermatozoa or 91% of the dry lipide-free mass of the head nuclei. This would mean that but little space in the sperm head can be allocated to nuclear material other than nucleoprotamine. On the other hand, Stedman and Stedman (282,283) claim that in sperm heads of salmon the nucleoprotamine does not account for more than 70% of the dry, lipide-free material, the remainder consisting to a large extent of a nonbasic protein "chromosomin," which contains tryptophan. "Chromosomin," however, must not be confused with "chromosin," a name given by Mirsky and Pollister (212) to a nucleoprotein complex extracted with 1 *M* sodium chloride from isolated cell nuclei of a great many organs including thymus, liver, spleen, pancreas, brain, frog testes, and bacteria. This complex has been resolved into three components: desoxyribonucleic acid, histone, and a protein which contains tryptophan. Both "chromosin" and the nucleoprotamine from trout sperm, when prepared by extraction with 1 *M* sodium chloride, are of strikingly fibrous character somewhat like muscle myosin. However, their fibrous nature is due to the presence of polymerized desoxyribonucleic acid and bears therefore no real resemblance to myosin. This essential difference must be emphasized in view of the claims made from time to time that spermatozoa contain a protein analogous in properties to myosin. There may well be such protein in spermatozoa but it still remains to be discovered.

The relative abundance of data available for fish spermatozoa is in contrast to the meager information on the nucleoproteins of mammalian spermatozoa. However, what evidence there is justifies amply the conclusion that nucleic acid of the type of thymonucleic acid is present in large concentration in the heads of mammalian spermatozoa. Zittle and O'Dell (332) applied sonic treatment to bovine epididymal spermatozoa to fractionate them into heads and tails, and found that, of the 4% phosphorus contained in the heads, all can probably be accounted for as thymus type nucleic acid. The

tails, on the other hand, were found to contain a large amount of lipide (23%), which the authors regard as a possible constituent of the tail sheath.

Our own experience (196) regarding the partition of nucleic acid and certain other constituents between the sperm heads and tails is chiefly based on observations of ram spermatozoa disintegrated with fine glass beads in a rapid mechanical shaker. Such treatment resulted in complete disruption and disappearance of tail and mid-piece but had no visible effect on the sperm head, so much so that when the tailless heads were counted, their number was found to correspond exactly to that of spermatozoa originally present in the sperm suspension. The sperm heads, after being washed, were free from both acid-soluble and alcohol-ether-soluble phosphorus compounds but contained 4% nucleic acid phosphorus. This corresponds to 140 mg. phosphorus per 100 ml. ram semen. When further examined by several analytical methods (49,268,271), the bulk of the "nucleic acid phosphorus" was found to be alkali-soluble and was precipitated by acid, thus behaving like desoxyribonucleic acid. However, a small proportion of the alkali-soluble material was not precipitable by acid. It is hoped to identify the precise nature of both these fractions when more material becomes available for a chemical isolation.

### C. ROLE OF ADENOSINE TRIPHOSPHATE

Spermatozoa, obtained by centrifugation of whole semen, contain an appreciable quantity of an acid-soluble phosphorus compound which yields inorganic phosphate on seven minutes' hydrolysis in 1 *N* hydrochloric acid at 100°. Lardy, Hansen, and Phillips (142) first observed the occurrence of such a compound in bull sperm and suggested that it might be adenosine triphosphate (ATP). However, the possibility was not excluded that the substance might be an adenyl polyphosphate other than ATP or one of the inorganic polyphosphates such as occur, for example, in yeast and molds (184, 185), all of which are decomposed in seven minutes by hydrolysis with 1 *N* hydrochloric acid. In order to obtain more definite information with regard to the chemical nature of the readily hydrolyzable phosphorus compound, its isolation from ram spermatozoa was undertaken (187,189). The compound has been isolated

in the form of its barium salt and identified as ATP on the basis of the molecular ratio of  $\frac{1}{2}$  between ammonia liberated enzymically from the adenylyl amino group, and phosphorus liberated by seven minutes' acid hydrolysis.

The content of ATP in ram spermatozoa varies from 0.5 to 1.5 mg. ATP amino nitrogen or 2.2 to 6.6 mg. ATP readily hydrolyzable phosphorus per 100 ml. ram semen (187,189). The occurrence of ATP in ram and boar spermatozoa has also been reported by Ivanov and co-workers (119), who found that the compound they purified from the sperm induced contractions of muscle actomyosin threads in the same manner as ATP isolated from skeletal muscle.

Regarding the function of ATP in spermatozoa, this has been examined in some detail by several investigators (60,119,142,187-189), all of whom conclude that ATP plays an important role in the motility of spermatozoa. Using ram spermatozoa under various experimental conditions we have established that the decrease in the content of ATP invariably coincides with the impairment of their activity (188,189). Thus, for instance, in ram spermatozoa which have been deprived by washing of all glycolyzable substrate, both ATP and motility decrease simultaneously under anaerobic conditions. Yet, ATP and motility can be maintained anaerobically if the spermatozoa are provided with glycolyzable material. This correlation between the level of ATP and the activity of spermatozoa is due to the pivotal position of ATP as a coenzyme in the carbohydrate metabolism of spermatozoa; it will be discussed together with the glycolytic enzymes in Section IV. Here it may be noted, however, that, aerobically, washed spermatozoa are capable of survival for a certain period of time even in the absence of carbohydrate, and one finds that both motility and ATP are preserved in washed spermatozoa for a longer time in air than in nitrogen. According to Lardy, Hansen, and Phillips (142), the level of ATP is maintained aerobically, at least in the epididymal sperm, by a continuous synthesis which occurs at the expense of phospholipide oxidation; they are of the opinion that the epididymal spermatozoa become and remain motile in contact with air because of the oxidation of the phospholipide stores and that this oxidation is associated with an uptake of inorganic phosphate which makes possible the formation of an easily hydrolyzable ester, probably identical with ATP.



Apart from ATP, certain other acid-labile compounds may be present in the spermatozoa. The possibility that phosphocreatine may be one of them has been considered first by Eggleton and Eggleton (55) on the ground that testicular tissue contains a labile phosphorus fraction which corresponds in properties to phosphocreatine. The ability of spermatozoa to synthesize phosphocreatine from creatine and phosphopyruvic acid through the "Parnas reaction" (227) was investigated by Torres (293) on bovine epididymal spermatozoa. She claimed to have found bull spermatozoa to be definitely capable of such a synthesis but her claim has been refuted by Ivanov (117), who could not repeat the experiments. On the other hand, it should be recalled that creatine was shown to occur in the testes of many animals, including the sea urchin (*Strongylocentrotus*), and also that phosphocreatine was actually isolated in the form of its calcium salt from the testes of the carp (76). More recently, a note appeared by Wajzer and Brochart (307) indicating that mammalian spermatozoa contain phosphoarginine rather than phosphocreatine.

#### D. IMPORTANCE OF SEMINAL PLASMA

The secretions of the accessory glands of which the seminal plasma is made up form the natural environment and nutrient medium for the spermatozoa and at the same time provide protection and act as a fluid vehicle for the sperm cells. The part played by seminal plasma in the composition of whole semen does not end here. The seminal plasma is richly endowed with highly active enzymes among which the phosphatases are particularly conspicuous. Furthermore, it is essential in the processes of coagulation and liquefaction of semen. It influences in a marked, not yet fully understood manner, the motility of spermatozoa and is capable of exerting striking biodynamic effects upon blood pressure and smooth muscles (62,67,102,118). The chemical composition differs vastly from that of most other body fluids including blood plasma, exhibiting as it does, a remarkably high content of certain unusual proteins (74,251), citric acid (266), fructose (191), and phosphorylcholine (168). The high buffering capacity is yet another important property of the seminal plasma; this is of particular value to the

spermatozoa in view of their sensitivity to acid, which accumulates as lactic acid during their metabolism. The components of seminal plasma credited with most of the buffering capacity of semen are inorganic phosphate, bicarbonate, citrate, and protein (2,266,276, 280). Proteins or, at any rate, high molecular and nondialyzable constituents of the seminal plasma may be also involved in an as yet inexplicable effect which the dialyzed seminal plasma exerts on spermatozoa, by prolonging the life of the sperm cells. Material of such protective nature was demonstrated, for example, in the seminal plasma of the sea urchin *Arbacia punctulata* (92,93). It was found to maintain the respiratory activity and to prolong the fertilizing capacity of sea urchin spermatozoa for a considerable length of time, not by virtue of any nutritive value but through an influence on the surface of spermatozoa. It is interesting to take note, however, that, although the proteins of the seminal plasma differ so greatly both in function and in physical properties from those present in spermatozoa, the sperm and plasma do not seem to differ greatly in their amino acid composition. In the case of bull semen, for instance, a recent survey of amino acids has shown that, with the exception of arginine, tryptophan, and leucine, the amino acid composition of bovine seminal plasma is rather similar to that of bull spermatozoa (264).

In recent years increasing attention has been focussed on the remarkable effect exerted by the male sex hormone upon accessory glands of reproduction connected with the elaboration of seminal plasma. The male hormone influences profoundly the secretory capacity of the epididymis, prostate, seminal vesicle, and Cowper's gland, and consequently it determines not only the output of seminal plasma as a whole but the relative contribution to the final make-up of seminal plasma, by each single accessory gland. In the absence of male sex hormone, *e.g.*, in very young or in castrated animals, the development of the accessory glands of reproduction and their secretory power are either restricted or arrested altogether. In response, however, to injected or implanted testicular hormone the accessory organs of a castrate male soon begin to grow, and before long their cytological structure and secretory function fully assume the character of organs in a nongonadectomized animal. If at that point the hormone treatment is once more suspended, the accessory

organs regress again and the secretion of the seminal plasma is once more curtailed (197,216,218). It is essential in biochemical studies of semen to take into account the composite nature of the seminal plasma, as determined by the influence of the testicular hormone on each of the contributing accessory organs separately, but also the possibility cannot be ignored that the output and composition of seminal plasma may undergo considerable fluctuations in a perfectly normal male, because of physiological day-to-day variations in hormonal activity. Apart from the testes there are other endocrine glands, such as the anterior pituitary, adrenal cortex, or the thyroid, which must not be disregarded as potent factors intrinsically linked with the elaboration of semen, and more particularly with that of seminal plasma.

The complex nature of seminal plasma makes it desirable for future biochemical analysis of semen to develop separate lines of investigations on each of the accessory gland secretions. Some such attempts have already been launched. A notable example of the fruitfulness of such investigations is the recent work of Huggins and co-workers concerning the chemical composition of the secretions of the prostate and seminal vesicle (103,105,107,109,272). Studies of this kind are likely to elucidate the effect of seminal plasma on the fertilizing capacity of spermatozoa. In the past such an effect has been frequently denied on the ground that fertilization can be brought about by epididymal spermatozoa as well as by highly dilute semen or by spermatozoa separated from seminal plasma by centrifugation. However, recent investigations, particularly those of Chang (40,41), reveal the importance of seminal plasma for fertilization when the number of spermatozoa used for insemination is reduced to a minimum. Just as the fertilizing capacity of a small number of sperm cells is more satisfactory in the presence than in the absence of seminal plasma, so the metabolic activity of dilute sperm suspensions also reacts favorably to seminal plasma. This is borne out, for instance, by our observations on the effect of dilution on fructolysis in bull sperm, where it was found that dilution of sperm with seminal plasma has remarkably little effect on the fructolysis index, whereas the use of artificial isotonic diluents invariably causes a decline in the rate of fructose utilization (194).

### E. SULFHYDRYL LINKAGES

The intense nitroprusside reaction given by the sperm head is a striking phenomenon described by Brachet (29) in conjunction with his studies on the localization of sulfhydryl proteins in spermatocytes and spermatozoa of insects and amphibia. The involvement of sulfhydryl groupings and thereby of some enzymic activities in the spermatozoa has been envisaged recently by MacLeod (180) in connection with his wartime studies on the inhibition of metabolism and motility of human spermatozoa by arsenicals. He found that sulfhydryl compounds which act as antidotes against arsenicals are at the same time capable of restoring the motility of spermatozoa. This is interesting in view of the analytical data pertaining to ram (75) and bull spermatozoa (332,333) which revealed a high level of sulfur-containing amino acids. Two-thirds of the 1.6% sulfur present in bull sperm was accounted for as cystine plus cysteine, and the remainder as methionine. There seems to be, however, a clear-cut difference in the partition of the two amino acids between heads and tails. The sulfur ratio between cystine and methionine was found to change from 1.09 in the tails to 4.87 in the heads. A particularly high concentration of cystine was found in that part of the sperm material which remains as an insoluble residue after extraction with 0.01 *N* sodium hydroxide and 0.01 *N* sulfuric acid, and which supposedly represents the membrane of the sperm head. The assumption that a keratinlike protein forms the membrane is further strengthened by the solubility of the cystine-rich material in thioglycolic acid. So far, however, the amounts of available material have not been sufficient to allow complete purification of the "keratin." Another remarkable physical property of the sperm membrane is the extraordinary elasticity of the sperm head structure as demonstrated in the skillful experiments of Moench and Holt (213), who were able to hook the head of the human spermatozoon by means of a microsurgical needle and to stretch it very considerably.

### III.‡ Role of Enzymes

The enzyme systems, with their specific coenzymes and carriers involved in the sperm respiration and glycolysis, will be discussed later in connection with the intermediary metabolic processes in

spermatozoa. It may be helpful, however, to refer here briefly to some general aspects of the problem as well as to certain enzymes, evidently of considerable importance, which apparently participate in semen metabolism, although in a manner as yet not fully understood.

Little is known at present about the possible relationship between the enzymic activities of semen and the occurrence in sperm and seminal plasma of certain vitamins and microelements. A particularly high level in semen is characteristic of ascorbic acid: 12 mg. per 100 ml. in human semen; 3–8 mg. per 100 ml. in bull semen; up to 8 mg. per 100 ml. in guinea pig ejaculate (17,231,331). Much lower values were observed in animals with poor breeding capacity and those suffering from scurvy (331). Ascorbic acid was shown to be derived from the seminal vesicles and must thus be considered a constituent of seminal plasma rather than of spermatozoa (17). The thiamine, riboflavin, pantothenic acid, and niacin contents of fresh bull semen were found to be 0.089, 0.209, 0.371, and 0.363 mg. per 100 ml., respectively, and the concentration of all four vitamins was correlated with the number of spermatozoa; with the exception of pantothenic acid the vitamins of the B group were also stated to be correlated significantly with the initial motility of bull spermatozoa (305). Regarding the microelements present in semen, at least three—iron, copper, and zinc—were recognized as normal semen constituents (187). A substantial part of the total iron in spermatozoa is nonhemin iron; in bull sperm the nonhemin iron was estimated to be 60% of the total iron (335). However, with the exception of iron, part of which is associated with the sperm cytochrome, the form and function of the microelements in semen remain unknown at present.

#### A. COAGULATION AND LIQUEFACTION OF SEMEN

Although mammalian semen is delivered from the urethra in a liquid state, in many species it tends to coagulate shortly after ejaculation. This phenomenon is particularly well developed in rodents. In rat and guinea pig, for instance, soon after ejaculation the semen becomes a solid mass; in the vagina it forms the *bouchon vaginal*, a solid plug which prevents the outflow of semen from the vagina. A gelatination of semen can also be observed quite frequently

in other animals such as rabbit, stallion, and boar. A fresh ejaculate of boar which may be as much as 0.5 liter, when inspected shortly after delivery contains small lumps of gelatinous material resembling tapioca. On standing, however, the lumps increase quickly in size and in the end take up half or more of the entire ejaculate.

It is generally believed that the substrate for the formation of gel and of the vaginal plug consists in most species of some protein-like material secreted by the seminal vesicles (18,21,97,141,154,236, 285) but the catalyst responsible for the coagulation is not present in the vesicles themselves but comes into effective contact with its substrate only in the course of ejaculation. Camus and Gley (34) were the first to recognize the enzymic nature of the coagulating agent to which they gave the name "vesiculase." At first the prostate gland as a whole was credited by most investigators with the function of vesiculase formation. Later, however, it was shown by Walker (308) that the source of the coagulating enzyme, at least in rat and guinea pig, is the so-called "coagulating gland," a small glandular structure immediately adjacent to the seminal vesicle. The coagulating power of the secretory fluid obtained from the coagulating gland is such that one part of it is sufficient to clot well over 20,000 parts of the vesicular secretion. This effect can easily be demonstrated *in vitro* if the contents of the coagulating glands and seminal vesicles are collected in two separate test tubes, diluted with saline solution, filtered, and the two filtrates mixed together; provided that the dilution with saline was not excessive, the formation of coagulum sets in almost immediately and before long the tube may be inverted without any disturbance of the contents. This method was employed by Moore and Gallagher (220) to study the coagulation phenomenon with secretions obtained from guinea pig.

Very little is known now about the nature of either vesiculase or the protein in the seminal vesicle which provides the coagulable material. Similarly, little is known about the mechanism of semen coagulation except that it differs in several respects from the system which operates in blood coagulation. It should also be pointed out that the phenomenon of semen coagulation is not a general one, but restricted to certain species. The semen of bull and dog, for example, remains liquid; on the other hand, in some species, notably in man, there is considerable evidence that the phenomenon does occur but

only to a limited extent. Human semen coagulates immediately after ejaculation but a little later it liquefies again, and until that happens the motility of spermatozoa is negligible; for this reason a microscopic assay of human spermatozoa should be postponed as a rule until twenty or thirty minutes after the emission of semen (181,279). In human and also in dog semen, Huggins and Neal (108) have discovered the presence of an enzyme, fibrinogenase, which digests added fibrinogen and which, if added to blood, prevents the coagulation of blood. In addition, in semen of man and dog they found a second proteolytic enzyme, fibrinolysin, which liquefies blood fibrin. Both these enzymes may be in some way connected with the liquefaction of human semen. Both are derived from the prostate gland; fibrinogenase has some properties in common, but is not identical, with trypsin; fibrinolysin closely resembles the fibrinolytic enzyme formed by the hemolytic streptococci (110).

In addition to the enzymes vesiculase, fibrinogenase, and fibrinolysin, there are other factors present in semen which probably also affect the coagulation and liquefaction phenomena, such as citric acid. Human prostatic fluid heated in a water bath at 100°C. for thirty minutes still delays considerably blood coagulation but the delay can be abolished by adding calcium ions. The cause of the prolonged coagulation time of blood in the presence of the heat-inactivated prostatic fluid has been traced by Huggins and Neal (108) to the presence in human prostatic fluid of large concentrations of citric acid (up to 2.6%). Of course, this factor does not enter into play in dog semen, which is very poor in citric acid.

#### B. SPERM HYALURONIDASE

A group of agents, mostly enzymes, which in recent times acquired much prominence, is that comprising the various sperm-egg interacting substances. Their existence and possible role in egg fertilization received considerable attention already from such pioneers in the field of sex physiology as Lillie (162,163) and Loeb (164-166). As the result of prolonged controversy concerning "fertilizin" and "antifertilizin," certain fundamental facts have now emerged, especially in relation to certain substances and enzymes which enable the spermatozoon to penetrate the egg surroundings and to enter the egg protoplasm. Several such substances have been de-

scribed, *e.g.*, the "egg membrane lysin," which Tyler (299,301,302) prepared from the sperm of certain molluscs, the "jelly coat dissolving factor" of Hartmann and Schartau (87), and the "surface liquefying agent" of Runnström *et al.* (256-258) in sea urchins, the "cumulus dispersing factor" of Yamane (328) and Pincus (232, 233), and last, but not least, *hyaluronidase*.

A detailed account of the recent developments in studies on hyaluronidase is beyond the scope of this article, especially since these have been reviewed several times during the past few years (198,206,302). However, a short description of the principal features of sperm hyaluronidase will be made here to make possible an appraisal of the present position.

The early observations made by Hoffman and Duran-Reynals (100) and McClean (172,173) on the existence in mammalian testes and spermatozoa of a factor which increases the permeability of the skin to fluids injected intradermally were followed some years later by the work of Chain and Duthie (37,38), who showed that fairly pure preparations of testicular "spreading factor" also possess strong "hyaluronidase" activity, *i.e.*, that of the mucolytic enzyme first described by Meyer, Dubos, and Smythe (207), which leads to depolymerization of hyaluronic acid. In 1942, McClean and Rowlands (174) found that hyaluronidase, not only from testes and spermatozoa but also from bacteria and snake venom, was able to liquefy the viscous gel cementing the follicle cells around freshly ovulated rat ova, thereby acting in a manner similar to the cumulus-dispersing heat-labile factor described in rabbit spermatozoa some time previously by Pincus and Enzmann (233). Analogous results on the mouse were reported by Fekete and Duran-Reynals (64), who observed that the viscous gel of the cumulus responds to staining methods (purple metachromatic reaction with toluidine blue) in the same way as hyaluronic acid. The presence of a substance in sea urchin sperm, possibly related to hyaluronidase, which dissolves the jelly coat of sea urchin eggs, has recently been claimed by Monroy and Ruffo (214).

Hyaluronidase has been demonstrated in the testes and sperm of several mammalian species including man, bull, boar, rabbit, and rat, but it has not been found in birds and reptiles, where the ova are not surrounded by follicle cell cumuli (64,122,138,286,290,319). The



sperm hyaluronidase was shown to originate chiefly in the seminiferous epithelium of the testes. Although associated with spermatozoa and not with seminal plasma, hyaluronidase can be easily released by the sperm cell into the surrounding medium (94,123,228, 287,288). A few hours' freezing of an aqueous sperm suspension at  $-10^{\circ}\text{C}$ ., or 24 hours' standing at  $0^{\circ}$  has been found by Swyer (287,288) sufficient for hyaluronidase to pass completely into solution. But even if the spermatozoa are suspended in an isotonic medium, hyaluronidase diffuses from the sperm cells and the amount of released enzyme is roughly proportional to the logarithm of the volume of suspension for a constant number of sperm cells. However, when a certain amount of hyaluronidase has accumulated in the medium, it seems to prevent further leakage of the enzyme from the sperm. Regarding the mode of release, Perlman, Leonard, and Kurzrok (228) point out that the enzyme liberation by spermatozoa may be more an indication of a moribund cell population than a prerogative of live cells.

The possible participation of sperm hyaluronidase in the process of egg fertilization is still under discussion. One of the greatest mysteries concerning the phenomenon of fertilization has always been that, although the actual fertilization consists ultimately of the entry of a single spermatozoon into the egg protoplasm, this usually takes place only after the site of fertilization has been reached by a large number of spermatozoa. To ensure fertilization it is obviously necessary to use many thousands of spermatozoa for a single insemination, a circumstance clearly demonstrated in experiments on rabbits where 330,000 to 1,000,000 spermatozoa were shown to be required for the maximum fertility of a single doe (41,255,309). In addition, the denudation of an ovum from the follicular cells has also been claimed as a process which requires a large number of spermatozoa (232). This led McClean and Rowlands (174) to suggest that the presence of a high sperm concentration in the vicinity of the egg may be required to create around the ovum a concentration of hyaluronidase sufficient to induce a complete denudation of the egg as a condition to the entry of one sperm into the egg cell. This supposition was put to experimental test by Rowlands (255), who found that it is actually possible to fertilize a rabbit ovum with a subnormal number of spermatozoa provided

that the sperm suspension is supplemented with sperm hyaluronidase in the form of cell-free extracts from whole, dilute rabbit semen. Similar results were reported by Leonard and Kurzrok (157,158). These authors also set out to determine the effect of immunization of animals to hyaluronidase, to see if it prevents the follicular cells from being dispersed by the enzyme, and if an anti-hyaluronidase serum would inhibit the action of hyaluronidase *in vitro*. They found that the follicle cells of rat ova are readily dispersed by bull testis extracts regardless of whether or not the rats were immunized beforehand against bull testes extracts. However, dilute antiserum from rats immunized against bull hyaluronidase had a marked inhibitory effect on the activity of the enzyme toward the follicle cells *in vitro*, whereas normal rat serum of the same dilution was not effective. Hyaluronic acid derivatives which inhibit the viscosity-reducing activity of testis hyaluronidase *in vitro*, were found by Pincus, Pirie, and Chang (234) to check also the cumulus dispersing action of rabbit sperm and of sperm hyaluronidase *in vitro*.

Several investigators have been attracted recently by the possibility of therapeutic application of hyaluronidase in infertility (122, 138,287,290,319) and there have been some reports of successful treatment of human infertility of oligospermic origin by supplementing the deficient seminal hyaluronidase with bull hyaluronidase added to human semen. However, for the moment, the number of treated cases is too small to warrant any definite conclusions. In fact, the very existence of a causal link between the fertilizing capacity and hyaluronidase content of semen must still be regarded as rather uncertain. Purified hyaluronidase preparations were found by Chang (40) to be without any significant effect on the fertilizing capacity of rabbit semen *in vivo*; he points out that the earlier positive results were obtained mainly with hyaluronidase used in the form of filtrates or extracts from whole semen and might therefore be attributed to the beneficial effect of seminal plasma as such rather than to that of hyaluronidase. Similarly, the concept that large numbers of spermatozoa are needed to denude a single ovum from its cumulus has recently been questioned by Leonard, Perlman, and Kurzrok (159) and by Austin (4), who demonstrated clearly that there is no mass removal of the follicle cells prior to fertilization and that sperm penetration precedes the gross denudation of

the ovum in rat. Thus the mechanism whereby the ovum is freed from the cumulus remains for the time being rather obscure, and the possibility cannot be excluded that for the complete denudation of ova, at least *in vivo*, the mechanical action of the cilia of the oviduct or the action of some other tubal factor (232,233,289) may be equally or even more important than sperm hyaluronidase.

### C. SEMINAL PHOSPHATASES

Unlike hyaluronidase, which is chiefly associated with the sperm cells, the powerful phosphatase activity of semen is derived mainly from the seminal plasma. Most of the work on seminal phosphatases has been carried out with human semen. Kutscher and Wolbergs (139) discovered the high phosphatase activity of the human semen and were the first to point out that the prostate gland is the main source of seminal phosphatase. The range of activity of the prostatic phosphatase was defined by the discoverers as that of an enzyme which acts optimally at a slightly *acid pH*, splitting equally well  $\alpha$ - and  $\beta$ -phosphoglycerol but which has only a slight effect on diphosphohexose and on pyrophosphate. Together with other constituents of seminal plasma, the acid phosphatase ranks as a chemical secondary characteristic of the male sex. Its level in the prostate is very low in childhood but increases rapidly during puberty; in immature monkeys the formation of the enzyme in the prostate can be enhanced promptly by androgen administration; a certain correlation appears to exist between the androgen activity in man and the level of acid phosphatase in semen (59,80,82,84). Normally, the acid phosphatase of the prostate does not enter the blood stream. However, large amounts of it appear in the blood plasma as a result of malignant growth in the prostate or metastases of prostatic cancer in the skeleton; injection of androgen raises the acid phosphatase in blood plasma; castration and injections of estrogen cause a fall (81,83,104,133,316). Histochemical studies by Gomori (69) have shown that in the human prostate the acid phosphatase is localized in the glandular epithelium; the prostate contains also an alkaline phosphatase, which, however, occurs chiefly in the walls of capillaries.

An important new development in the studies on phosphatase, pertaining to the physiological role of this enzyme in semen, has

been the discovery by Lundquist (168,169) that freshly ejaculated human semen contains phosphorylcholine which originates in the seminal vesicles but on ejaculation is mixed with the phosphatase-rich prostate secretion and split rapidly into choline and inorganic phosphate. According to Lundquist, the splitting of phosphorylcholine may be a true physiological function of the prostatic phosphatase. It certainly accounts for the fact that, whereas in freshly ejaculated human semen the amount of inorganic phosphate is only about 10 mg. phosphorus per 100 ml., twenty minutes after ejaculation it rises to 64 mg. per 100 ml., corresponding to the breakdown of the major part of total organic, acid-soluble phosphorus originally contained in fresh seminal plasma.

However, the fact must not be overlooked that the prostate is by no means the sole source of phosphatase in semen, indeed, the so-called seminal phosphatase is probably a mixture of several dephosphorylating enzymes which come partly from the prostate and partly from other organs, particularly the seminal vesicle (193). Reis (245-247) found that human seminal plasma has a high phosphatase activity toward certain nucleotides such as inosinic acid, adenylic acid, and yeast adenine nucleotide. He has shown that among the seminal phosphatases there is one specifically concerned with the dephosphorylation of adenylic acid. This enzyme, the "5-nucleotidase" as he called it, was shown to be highly active against adenylic acid but completely inactive toward ATP and adenine nucleotide. Using bull semen, we were able to confirm the existence of a highly active 5-nucleotidase (187,193). In bull seminal plasma the activity found could be expressed by the ratio:

$$\frac{\text{moles phosphorus split from adenylic acid}}{\text{moles phosphorus split from } \beta\text{-phosphoglycerol}} = \frac{300}{1}$$

The potency of the seminal plasma can be judged by the fact that from sodium adenylate containing 160  $\mu$ g. phosphorus added to 0.001 ml. seminal plasma, 140  $\mu$ g. phosphorus was liberated as inorganic phosphate after one hour's incubation at 37°. Expressed in phosphatase units, the nucleotidase activity in the bull seminal plasma is 3000-5000 as compared to 30 in brain, 2 in skeletal muscle, and 0.05 in normal serum. Yeast adenine nucleotide is

dephosphorylated by bull seminal plasma much more slowly than muscle adenylic acid. ATP, on the other hand, occupies an intermediary position between the two nucleotides.

Although it is the seminal plasma which is endowed with most of the phosphatase activity, the spermatozoa are by no means phosphatase-free. Bull spermatozoa, for instance, even after repeated washing, still exhibit a remarkably high phosphatase activity, particularly toward ATP. The 5-nucleotidase, on the other hand, is much weaker in the spermatozoa than in the seminal plasma. The position is analogous in the semen of boar and ram, but the activity of the seminal plasma phosphatases is not nearly as pronounced as in bull. On the other hand, the activity of ram spermatozoa toward ATP is of the same order as that of the equivalent number of bull spermatozoa (187).

These facts, and especially those relating to the very high phosphatase content of human seminal plasma as distinct from the relatively low phosphatase activity in ram, have a direct bearing on metabolic studies on spermatozoa. Actually, these circumstances primarily induced us to choose ram semen in preference to other species as an object for studies of the phosphorylating mechanism in sperm glycolysis (188,189,191). Ram semen is particularly suitable for such studies because of its exceptionally high density and the remarkably high resistance of ram spermatozoa to experimental procedures such as centrifugation and washing; in addition, the comparatively weak phosphatase of ram seminal plasma can easily be removed by centrifugation and washing of sperm. This eliminates the danger of interference from the phosphatase toward phosphorylated compounds, which can be expected to be formed in the course of the intermediary reactions of sperm glycolysis. Using ram semen, the author (188) was able to demonstrate various phosphorylated esters as intermediary products of sperm glycolysis. He experienced none of the difficulties reported by investigators on human semen (183), which were probably due to the fact that human spermatozoa are neither as resistant to centrifugation as those of ram, nor is it easy to free the former effectively from the adhering plasma phosphatase. To all this is added the difficult task of securing sufficiently concentrated sperm suspensions from human semen, the sperm density of which is about thirty times lower than in ram semen.

#### IV. Fructose and Fructolysis

The presence of oxygen is not an essential condition for the survival of spermatozoa. This was pointed out by Walton, Hammond, and Asdell (314) when they found that mammalian semen can be successfully stored in absence of air. Shortly afterward, Ivanov (114) showed that dog spermatozoa suspended in an isotonic solution of glucose and phosphate retained their motility when their respiration has been abolished either by poisoning with cyanide or by replacing the air with hydrogen. These observations were considerably extended by Redenz (244), who found that the presence of glucose, fructose, or mannose but not that of glycogen, sucrose, or lactose is beneficial to sperm motility, especially under anaerobic conditions, and that the anaerobic survival of bull spermatozoa, at least those obtained directly from the epididymis, is dependent on the addition of monosugars, which can be glycolyzed by the sperm cells to lactic acid. Soon it became obvious, following a series of important investigations (45,96,115,143,171,176,177,215,250,276), that the metabolism of spermatozoa in several mammalian species, including man, ram and bull, is predominantly of a glycolytic character. In the course of these studies it was also noticed that in many instances the rate of glycolysis in semen was related in a rather strict and quantitative manner to the number of sperm cells and their motility, so much so that sperm glycolysis was suggested as a basis for the evaluation of sperm quality. In recent times the value of sperm glycolysis for the appraisal of sperm quality was stressed and discussed in all its aspects by Salisbury (259).

##### A. FRUCTOSE AS A NORMAL CONSTITUENT OF SEMINAL PLASMA

The earlier researches on sperm glycolysis brought out the fact that the spermatozoa obtained directly from the epididymis as well as those obtained from ejaculated semen by centrifugation and washing exhibit in certain aspects a behavior strikingly different from that of whole semen. Whereas epididymal, or ejaculated and then washed, spermatozoa can survive anaerobically only in presence of added glucose or some other glycolyzable carbohydrate, whole semen requires no further addition of sugar for the survival of sperm cells, which continues at the expense of the reducing and yeast-fermentable carbohydrate present in the seminal plasma. For a long

time, however, the nature of this carbohydrate remained unknown. In the literature dealing with this subject (19,52,66,106,109,132,175,182,215,262,276) it has been described either as glucose or simply as the "reducing sugar of semen," and the only reference to a probable occurrence of fructose in semen is found in an early paper by Yamada (327), who made a general survey of human tissues and body fluids for the presence of fructose by means of a color reaction with "Cryogénine" (the antipyretic drug "Cryogénine Lumière"). The problem was finally settled by chemical investigations, which showed that the sugar in semen is not glucose but D-fructose (190-192).

Brief reference may be made here to the events which led the author to the discovery of fructose in semen, since these bring out rather well the general value of isolated enzymes as useful tools in the unraveling of chemical organic structures. During some earlier experiments on the purification of metaphosphatase and metaphosphate from the mycelia and culture media of *Aspergillus niger* (184-186), a preparation of glucose oxidase was obtained as a by-product which, although crude, oxidized specifically glucose ( $Q_{01} = 1000$ ) but was devoid of activity toward either fructose or mannose. In order to confirm the earlier claims that seminal plasma, particularly that of bull, is rich in glucose, the reducing sugar value of 0.1 ml. bull seminal plasma was determined before and after one hour's aerobic incubation with 1 mg. of glucose oxidase preparation. The result was that there was no change in the reducing value, and yet, when the experiment was repeated with glucose artificially added to the seminal plasma, the added glucose was found to be effectively removed by the mold enzyme within a few minutes. Similar results were obtained when, instead of the crude enzyme, a highly purified preparation of glucose oxidase was used as employed by Keilin and Hartree (129) in their study of glucose-producing biological systems. Next, a large sample of seminal plasma was used for the preparation of phenylosazone and this yielded a crystalline product which melted at  $205^{\circ}$ , thus pointing toward the presence of either fructose or mannose. At this point, the Seliwanoff reaction was performed in seminal plasma. It was strongly positive.

In connection with the color reactions for fructose it must be remembered that none of the substances which produce color reac-

tions with fructose, such as resorcinol (273), diphenylamine (113), naphthol (235), cryogenin (327), or various indole derivatives (124, 241,291,292), can be regarded as specific for fructose, since they give positive results not only with fructose as such but also with a variety of other substances such as the Neuberg ester, Harden-Young ester, sucrose, methylglyoxal, dihydroxyacetone, and numerous other ketose derivatives (191,195,224). It would be, therefore, entirely inadmissible to claim the presence of fructose in semen, or in any other biological material, on the basis of the color reaction alone without preceding chemical proof that the compound responsible for the color test is in fact fructose. However, in the case of semen we have provided satisfactory evidence that the color reaction, as used for the assay of fructose, strictly corresponds to the content of this sugar alone (191,195). Proof to that effect was furnished when it was shown that (a) fructose occurs in the semen in free form and accounts for the whole of the yeast-fermentable material which yields the color reaction, (b) there are no other substances in seminal plasma which would yield the Seliwanoff reaction, and (c) fructose can be purified from semen and identified by its reducing value, specific optical activity, and the preparation of the crystalline methylphenylosazone.

The level of fructose in semen varies from one species to another. It ranges from a few milligram per cent in boar and stallion to several hundred milligram per cent in bull, ram, rabbit, and man; in bull the concentration may exceed 1000 milligram per cent. However, even within the same species there are very large individual differences. Moreover, considerable fluctuations are observed in the semen collected from the same ram on different occasions. If two collections of semen from the same animal are made in quick succession, one finds that the concentration of fructose is mostly higher in the second ejaculate than in the first. In some species such as man, boar, and stallion, it is possible to apply in the analysis of semen the so-called "split ejaculate method," *i.e.*, to collect and analyze several separate fractions of a single ejaculate according to time of delivery from the urethra. In such conditions one finds that fructose is not equally distributed in all portions of the ejaculate but is highest in the last fraction. This is the fraction which, at least in man, is representative mostly of the seminal vesicle secretion.



## B. SEMINAL VESICLE AS FRUCTOSE-PRODUCING ORGAN

Huggins and Johnson (106) were the first to show that the high concentration of reducing sugar in human semen is due principally to the secretion of the seminal vesicles and not the prostate. Similar observations were made by Bernstein (20) on bull, by McKenzie, Miller, and Bauguess (175) on boar, and by Moore and Mayer (215) on ram. The recent identification of fructose as the sugar of semen made it possible for us to pursue a more detailed investigation of the seminal sugar.

If the high content of fructose in semen were connected with the presence of spermatozoa as such, one would expect in the testes and in the epididymis an even higher concentration of fructose. However, the epididymal semen, *i.e.*, the thick mass of spermatozoa obtained directly from the epididymis, gave a barely perceptible Seliwanoff reaction, indicating that the site of fructose formation in the male body must be situated along the generative passage between the epididymis and the urethral outlet. An extensive study of fructose distribution in various accessory organs showed that in many species fructose originates chiefly in the seminal vesicle (191, 193-195). Up to a point, therefore, one could use the estimation of fructose in semen to assess the relative contribution made by the seminal vesicle toward the make-up of the whole semen. We soon found, however, that this would be only an approximate and not too accurate indicator because, although the seminal vesicles produce the bulk of fructose, they are by no means the sole source of the seminal sugar. In both bull and ram, fructose was also found in the ampullae, where it is probably produced by the ampullar glands. In rat it was found in the coagulating gland and in the dorsal prostate. The rabbit provides an interesting instance, since it is an animal which has no seminal vesicle and yet was found to secrete fructose in semen (191). In order to solve this discrepancy Davies and Mann (50,51) undertook a joint anatomical and chemical study of the various parts of the male reproductive system of the rabbit, from the early development stage up to the time of sexual maturation. This study has established that the *glandula vesicularis* of rabbit is both morphologically and functionally analogous to the seminal vesicle of other animals. However, apart from this gland, fructose was also found in the ampullae as well as in the "prostate

proper" of the rabbit in a manner resembling conditions described by Humphrey and Mann (111,112) in the rat. On the other hand, in the dog, which has neither a seminal vesicle nor a structure that would correspond to this gland, only a trace of fructose was found in the semen (194).

In view of the fact that the site of fructose generation is distinct from that of sperm formation, it is not surprising that in the whole semen there is no constant ratio between fructose concentration and sperm density. The absence of rigid co-ordination between the two constituents of semen is particularly noticeable in an experiment in which an animal is subjected to a so-called "exhaustion test." This test, which Walton and Edwards (313) developed some years ago in conjunction with their study of bull semen, depends on the analysis of a series of semen samples collected from a single animal at frequent, short intervals. The results obtained with fructose in the "exhaustion test" (194,195) are shown in Table II. In this instance

TABLE II  
EFFECT OF FREQUENCY OF COLLECTION ON SPERM DENSITY AND ON CONCENTRATION OF FRUCTOSE AND LACTIC ACID IN FRESH BULL SEMEN (194)

Ejaculate No.	Time of collection, min.	Volume of ejaculate, ml.	Sperm density, thousands/ $\mu$ l semen	Fructose, mg./100 ml. semen	Lactic acid, mg./100 ml. semen
1	0	4.2	1664	760	35
2	10	3.9	680	890	23
3	18	3.7	254	900	22
4	28	3.7	648	750	22
5	38	3.4	135	820	23
6	45	3.5	342	820	22
7	55	2.7	390	630	8
8	63	2.9	98	690	29

the bull yielded eight ejaculates within 63 minutes at 7-10 minute intervals. The effect of the multiple collection is most obvious in the decrease of sperm density from 1,664,000 cells per microliter in the first ejaculate to 98,000 cells per microliter in the last ejaculate. This fall, however, was not accompanied by a corresponding decrease in fructose concentration, which differed but little in the first and the last ejaculate. In connection with the above test, however, it should be pointed out that the seminal vesicle (seminal

gland) of bull differs from the analogous organ in man and some other mammals by its exceptionally high capacity for storing large quantities of a fructose-rich secretory fluid. In some bulls (slaughter house material) we were able to recover up to 50 ml. of secretory fluid from the seminal vesicle with as much as 1.5% fructose.

### C. FRUCTOLYSIS AS SOURCE OF ENERGY FOR SPERMATOZOA

On the basis of recent studies (191,194,200) the function of fructose in semen would seem to be to provide a readily utilizable store of energy for the survival of motile spermatozoa. At the site of origin, in the testis and in the epididymis, the spermatozoa are immotile. However, when they traverse the male generative passages

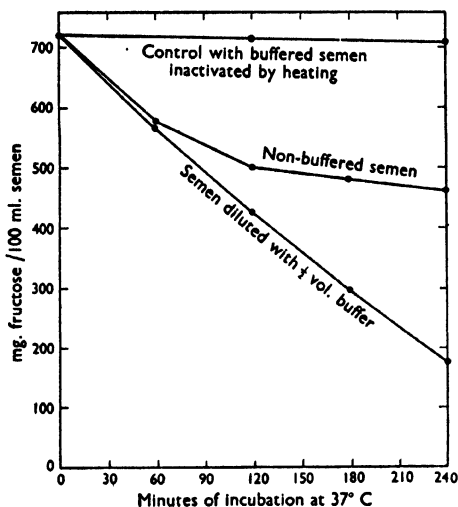


Fig. 1. Fructolysis in bull semen incubated at 37°C. (195).

from the testes onward they are enriched by the secretions of the accessory organs of reproduction and they assume a high degree of motility, which requires considerable metabolic energy. This is made available, at least in those species which contain fructose in the seminal plasma, through chemical reactions which constitute the process of sperm fructolysis.

The incubation of freshly ejaculated whole semen is followed by a progressive fall in fructose, accompanied by the accumulation of lactic acid. The rate of fructolysis is greater in nitrogen than in air, and the ratio between lactic acid produced and fructose used up is appreciably higher in nitrogen than in air, due to the oxidation of lactic acid in oxygen. In semen of high sperm density as, for instance, in ram, the fructose reserve may run out within an hour or so, at 37°. If fructolysis is allowed to proceed until fructose is practically exhausted and if at this point fresh fructose is added, the spermatozoa will continue their glycolytic activity and vigorously produce lactic acid. However, in order to demonstrate this effect of fructose it is essential to ensure an adequate supply of buffer to the semen, since otherwise the spermatozoa become immotile due to acidification of semen, which is the outcome of their own fructolysis. With the addition of a suitable quantity of buffer, the disappearance of fructose can be shown to follow an almost linear course until practically all the sugar is used up (Fig. 1). On this basis a method has been worked out for a quick, simple, and yet accurate assessment of sperm fructolysis (194,195), which, it is hoped, will replace the older, more tedious, assays of sperm glycolysis.

We are confronted with the interesting question as to why the male organism should possess fructose and not, say, glucose or glycogen, as the natural sugar of semen, particularly in view of the fact that, although fructose is the sole and "physiological" substrate in whole semen, yet sperm cells washed free from seminal plasma are capable of utilizing added glucose to the same extent as added fructose (Fig. 2). In this respect the behavior of spermatozoa toward fructose is unlike that of the majority of other animal tissues, which anaerobically form lactic acid from glucose much more readily than from fructose. The contrast is particularly marked when spermatozoa are compared with the seminal vesicle. This organ, which is the store of seminal fructose, has been shown by Mann and Lutwak-Mann (200) to be capable of utilizing anaerobically only glucose but not fructose. It is conceivable that the preference of spermatozoa for fructose aids their survival, by ruling out substrate competition from other animal tissues. It is worthwhile to recall here other instances which create a similar problem, for example, the occurrence of fructose in fetuses (5) and insect larvae (160), and that of lactose in milk (125). Another possible interpre-

tation for the occurrence of fructose in semen may be sought in the intimate relationship between seminal fructose and the male sex hormone (202). This will be discussed later.

The ability of washed spermatozoa to make equal use of added fructose and glucose (or mannose) probably has its foundation in the fact that all three sugars enter the glycolysis by way of the

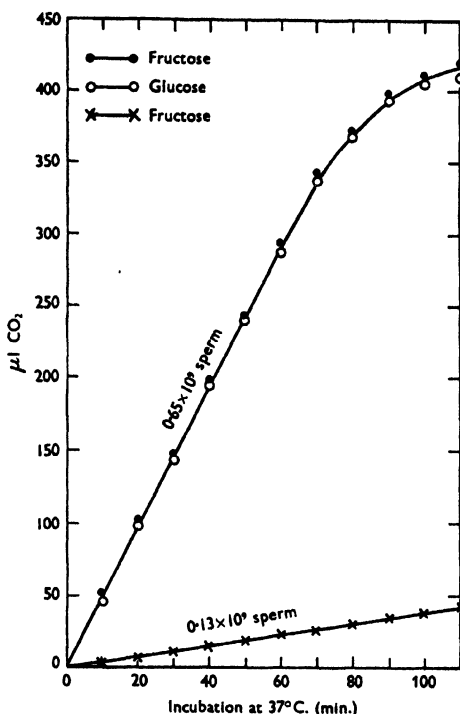


Fig. 2. Anaerobic fructolysis and glucolysis by washed ram sperm (200).

same enzymic reaction with ATP. Under the influence of the sperm hexokinase half the readily hydrolyzable phosphate of ATP is rapidly esterified, and 6-monophosphohexose (hexose-6-phosphate) is formed to the same extent with glucose, fructose, and mannose (188,191). From 6-monophosphohexose the glycolysis proceeds through diphosphofructose (fructose diphosphate), phosphotriose (triose phosphate), phosphoglyceric acid, and pyruvic acid to lactic

acid, and each of these intermediary products can be demonstrated under suitable experimental conditions with the use of washed sperm suspensions and the necessary reactants. The reconstitution of ATP, split during the initial stages of glycolysis, is accomplished by the reaction between phosphopyruvic acid and adenylic acid and by the reaction between inorganic phosphate and adenylic acid, which forms part of the phosphorylative oxidation of phosphotriose (188); the latter requires cozymase (DPN), which, like ATP, is a normal constituent of spermatozoa (324).

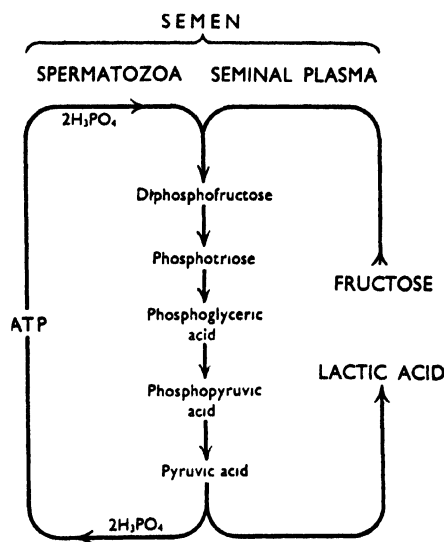


Fig. 3. Scheme of fructolysis in semen.

All intermediary steps in the glycolysis of spermatozoa were demonstrated with intact live sperm cells (188); there is little doubt, therefore, about their occurrence in the sperm under physiological conditions. Objections are occasionally raised against phosphorylation experiments carried out with cell-free tissue extracts or purified enzyme systems, the charge being that such results do not truly depict the state of affairs inside the living cell. In this respect the experimental results with sperm glycolysis were not only profitable *per se* but provided in addition welcome evidence to

strengthen the belief that phosphorylation in living cells runs a course analogous to that demonstrable by means of cell-free enzyme systems (199). The study of intermediary carbohydrate metabolism in spermatozoa also lent further support to the previously mentioned concept that ATP represents the link between the activity of spermatozoa and glycolysis. The content of ATP, and ultimately the anaerobic survival of the spermatozoa, both depend on the maintenance of sperm glycolysis. A schematic representation of the events in whole semen, leading from fructose to lactic acid, is shown in Figure 3.

#### D. ROLE OF MALE SEX HORMONE

In the past the fundamental anatomical and cytological studies provided the data on which is built our knowledge of the dependence existing between the activity of the male sex hormone and the functional state of male accessory organs of reproduction. These researches have shown that the weight, size, cytological appearance, and secretory function of certain organs such as epididymis, seminal vesicle, and prostate are strictly dependent on the secretion of the male sex hormone by the testes, and that postcastrate retrogressive changes in accessory glands can be prevented or reversed by injections of the testicular hormone, thus serving as "indicator tests" for the male sex hormone (53,167,208,217,219,221,222,238,239,336).

An admirable account of the existing indices, and their comparative value in the detection of male sex hormone, is contained in an article by Moore (216), where the following methods are listed: (1) *Capon comb growth test*, which depends on the growth response of the comb of a capon following subcutaneous injections of the male hormone; (2) *Spermatozoon motility test*, based on the observation that spermatozoa contained in an isolated epididymis show a longer persistence of viability (a higher capacity to produce movements on dilution with isotonic sodium chloride) when the male hormone is present (presumably this phenomenon is due to hormone stimulation of epididymal cells to secrete some substance necessary for preservation of epididymal spermatozoa); (3) *Electrical ejaculation test*, where seminal discharge and, consequently, the coagulation of semen are induced in guinea pig by electric stimulation; (4) *Seminal vesicle cytology test*, based on the typical changes which

occur in the secretory epithelium of rat seminal vesicle as a result of castration and subsequent treatment with male sex hormone; (5) *Prostate cytology test*, depending on similar changes in the rat prostate; and (6) *Couper's gland test* and (7) *Vas deferens test*, both similar to the other cytological methods.

To the indicator tests for the male sex hormone has recently been added the "fructose test," a reaction described by Mann and Parsons (202), based on the finding that the activity of testicular hormone in the male is reflected in the capacity of the accessory organs to produce and secrete fructose. In experiments on rabbits it was shown that fructose disappears almost completely within two weeks after castration and also that the postcastrate fall in the level of fructose can be prevented or, if already developed, restored by the implantation of testosterone (Fig. 4). The test can be carried out in two ways, by the chemical analysis of the seminal fluid collected by means of an artificial vagina from an intact animal, or by the analysis of accessory organs of reproduction obtained from the animal by dissection. Whichever is chosen, two further modifications are possible. In the so-called "maintenance test," the hormone treatment commences immediately after castration, whereby the accessory glands are maintained throughout the whole experiment in a state of complete or partial secretory activity. The alternative is the "regeneration test," in which the animal is left after castration for as long as may be required for the complete disappearance of fructose in semen, and then subjected to treatment with testicular hormone.

The special advantage of the "fructose test" is its simplicity and sensitivity. This was amply demonstrated by the results of the "Compton experiment" recently described by Mann, Davies, and Humphrey (197). For this experiment six bull calves were used. These were castrated when a few weeks old, *i.e.*, at an age prior to the appearance of fructose in the seminal glands. Seven months later two of the castrated animals had testosterone pellets implanted subcutaneously, whereas the remaining four were left untreated. Four weeks later all six animals were slaughtered and their seminal glands analyzed both chemically and histologically. The chemical analysis revealed a high content of fructose in the seminal glands in response to the four weeks' treatment, as against a negligible fructose level in the untreated castrated animals. In comparison,



however, with the unequivocal chemical changes, the evidence of functional recovery in the seminal glands, as assessed by the histological methods, was practically imperceptible.

Experiments on rabbits and bull calves have established the fact that in young developing animals fructose appears in the accessory glands at an early stage when the testes are still incapable of producing any live spermatozoa. This, together with the fact that the

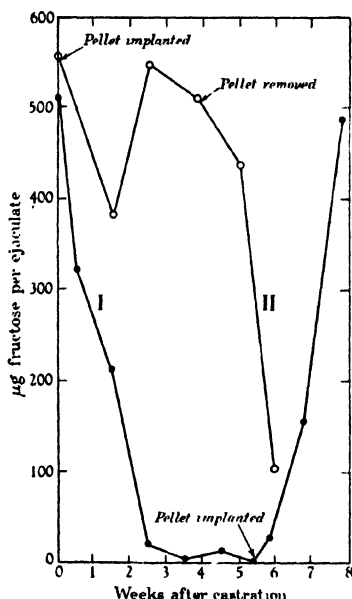


Fig. 4. Postcastrate fall and testosterone-induced rise of seminal fructose in rabbit (202).

formation of seminal fructose depends upon the testicular hormone, must be interpreted as evidence that the testicular hormone begins to function in the male body some time before actual spermatogenesis. At the same time, when the first mature spermatozoa make their appearance in the male generative tract, they can draw freely upon the fructose reserve. In view of the nutritive value of fructose to the sperm cells it would appear that a most essential function of the male sex hormone must be to stimulate metabolic processes

responsible for the formation of nutrient material in the form of seminal sugar. This concept has received strong additional evidence from some recent experiments, carried out by Mann, Lutwak-Mann, and Price (201). Using subcutaneous transplants of seminal vesicle and coagulating gland tissue in rat, they showed that such transplants are capable of secreting fructose and citric acid in response to testosterone, in complete anatomical separation from the rest of the reproductive tract. The most interesting fact concerning these experiments was that the growth of such grafts and their chemical secretory function took place not only in male rats but also in female rats provided these were treated with testosterone.

## V. Respiration

### A. MEASUREMENT OF OXYGEN CONSUMPTION IN SEMEN

In the presence of oxygen, spermatozoa show considerable respiratory activity, which is correlated significantly with both the density and motility of spermatozoa. Measurements of respiration have been made both with human spermatozoa (176,177,278) as well as with those of several higher animals including bull (96,142,146,150, 244,296,313,326), ram (42,45,46,152,188), boar (325), dog (22,114), fox (22), cock (146,323), and small laboratory animals (35,146, 326). The results of measurements of sperm respiration are customarily expressed in terms of  $Z_{O_2}$ , a coefficient which is introduced by Redenz (244) to denote the oxygen consumption in microliters oxygen taken up by  $10^8$  sperm cells in one hour;  $Z_{O_2}$  values reported by Lardy and Phillips (146) for bull, cock, rabbit, and ram spermatozoa are 21, 7, 11, and 22, respectively. The use of  $Z_{O_2}$  is preferable to  $Q_{O_2}$ , which involves washing spermatozoa with water and determining dry weight; the average  $Q_{O_2}$  of ram sperm obtained in this manner is about 8.

Investigators generally agree that the oxygen uptake as measured in whole semen must be almost entirely attributed to spermatozoa. It has been stated, however, that in some instances, notably in human semen, the bulk of the oxygen is consumed by the seminal plasma and not by the spermatozoa (177,250,330). According to Zeller (330), most of the oxygen consumption of human semen is due to the presence in the seminal plasma of the diamine oxidase, which can oxidize spermine as one of its substrates. However, in

view of the low sperm density of human semen, further experimental details are required before human spermatozoa can be pronounced nonrespiring cells. Much more definite are the results obtained with the use of animal semen. For example, in whole ejaculated semen of ram separated by centrifugation and washing with Ringer solution into spermatozoa and cell-free supernatant fluid, one finds the respiratory activity confined to the sperm cells (152,188). It is possible, however, that special occasions may arise when some oxidizable material other than sperm utilizes oxygen—for example, some cornified cells or tissue debris. Such cellular material was shown by Bishop (22) to contribute quite substantially to the large oxygen consumption of fox semen, and he traced its origin to the epididymis. The presence of a heat-stable and non-dialyzable factor in bull epididymal secretion was held responsible by Henle and Zittle (96) for part of the oxygen uptake observed in sperm obtained directly from the epididymis of the bull. In this connection, however, strong emphasis must be laid on the difference in the results obtained by measurements of respiration in epididymal as against ejaculated sperm. Since spermatozoa undergo a so-called “ripening process” in the epididymis and differ morphologically from the mature sperm cells of normal ejaculated semen, it is not surprising to find distinct metabolic characteristics for epididymal and ejaculated sperm. According to Henle and Zittle (96), bovine epididymal spermatozoa possess a lower endogenous respiration than the ejaculated sperm. Moreover, it has been reported (142) that the storage for a day or two of excised epididymis in the refrigerator leads to increased endogenous respiration rate of the sperm cells, possibly an outcome of maturation of spermatozoa in the epididymis. However, to fathom the differences between epididymal and ejaculated sperm much further work is needed. In particular, more evidence is required concerning the alleged difference in behavior toward glucose, which in bull was found to check the respiration of ejaculated sperm, but to enhance the respiration of spermatozoa obtained from the epididymis (96,143).

Another important factor worth considering in the respiration of mammalian sperm is the strongly adverse effect of dilution on the motility, viability, and metabolism of spermatozoa (41,43,58,200). This effect is particularly striking with 0.9% sodium chloride.

According to Milovanov, however, the spermatozoa are not all equally sensitive to dilution with 0.9% sodium chloride and those with higher "potential fertility" are more resistant than the others, so much so that he finds it possible to use the "resistance test" in assaying the value of semen specimens for artificial insemination (210).

Much interesting work has been done on the respiration of spermatozoa in lower animal species, particularly in sea urchin semen which, because of its close dependence on respiration and at the same time very low glycolytic activity, is particularly well suited for such investigations. Ripe sea urchin spermatozoa are motionless so long as they remain undiluted. However, when shed or artificially diluted with sea water they become intensely motile and at the same time their respiration increases. A quantitative analysis of this "dilution effect" was undertaken by Gray (71-73), who found that the level of activity exhibited immediately after activation with sea water depends on the degree of dilution and, furthermore, that the total mechanical activity is correlated in a striking manner with the rate at which the cells absorb oxygen. He observed also that a high respiratory rate such as exhibited by sperm diluted with sea water is of comparatively short duration and declines almost from the beginning of active life, whereas in sperm diluted with "egg water," i.e., with sea water previously exposed to contact with ripe sea urchin eggs, the respiratory activity is maintained for a long time or, in some species, even increased above the initial level. Much work has been done since on the activation phenomena in sea urchin semen, showing that there are several substances capable of stimulating sperm respiration, e.g., thyroxine (35,149) and various substituted nitro- and halophenols (149,303). However, the nature of the substance responsible for the activating effect of egg water remains still unsettled. It is thought by Hartmann, Schartau, Kuhn, and Wallenfels (88) to be identical with echinochrome but this claim has been seriously challenged by Tyler (300) and Cornman (47,48). Several theories have also been advanced to explain the lack of movement of sea urchin spermatozoa in undiluted semen. A critical analysis of the various views has been undertaken recently by Rothschild (254), who considers the low tension of oxygen and not lack of space to move

(allelostasis) as the main cause for the absence of sperm movement in the undiluted semen. He was able to demonstrate that sea urchin spermatozoa can be made motile in undiluted semen by increasing the oxygen tension but lose the motility when oxygen is replaced by nitrogen. Incidentally, this experiment provides also a fresh illustration of the old observation by Barron (7) that sea urchin spermatozoa, unlike those of mammals, cannot move in the absence of oxygen.

### B. CYTOCHROME

Keilin (126,127) has shown that the respiration of living cells is dependent on the presence of the cytochrome-cytochrome oxidase system. As long ago as 1925, he pointed out that among all the organs of a perfused frog two are distinguished by the highest concentration of cytochrome, heart and testis (126). Some years later Brachet (28) reported the presence of cytochrome in frog sperm obtained from the seminal vesicle, and Ball and Meyerhof (6) found cytochrome in sea urchin spermatozoa. In more recent years, several attempts were made to obtain spectroscopic evidence for the occurrence of cytochrome in mammalian spermatozoa but all these were completely negative, and the functioning of the cytochrome system was deduced only indirectly from experiments based on the succinic dehydrogenase and indophenol oxidase activity of spermatozoa (145,178,334). In order to solve the problem, a spectroscopic study was undertaken with ram and bull semen which because of high cell density are far more suitable objects for a study of this kind than human semen. The instrument used in this examination was the microspectroscope, which is particularly convenient for direct spectroscopic observations on tissues because of its small dispersion and strong illumination. No difficulty whatever was experienced in observing the full spectrum of cytochrome in both whole semen as well as in washed suspensions of ram and bull spermatozoa. In each case strong bands of all three cytochromes, *a*, *b*, and *c*, were clearly visible (187,189).

The full range of activity of the cytochrome system in mammalian spermatozoa was demonstrated when it was shown that (1) the cytochrome components of sperm undergo oxidation and reduction according to prevailing experimental conditions, and (2) the band of cytochrome *a*, which is particularly strong in spermatozoa,

undergoes a typical change under the influence of carbon monoxide. The carbon monoxide compound formed in sperm (187,189) was found to be identical with the carbon monoxide compound of cytochrome oxidase or cytochrome  $a_3$  originally described in heart muscle preparations by Keilin and Hartree (128).

The evidence for the participation of the cytochrome system in sperm respiration was further strengthened by the work of Rothschild (252,254), who demonstrated that the oxygen uptake of sea urchin spermatozoa is inhibited by carbon monoxide, an effect completely reversed by white light (Fig. 5) but not by light of 548  $m\mu$  wave-

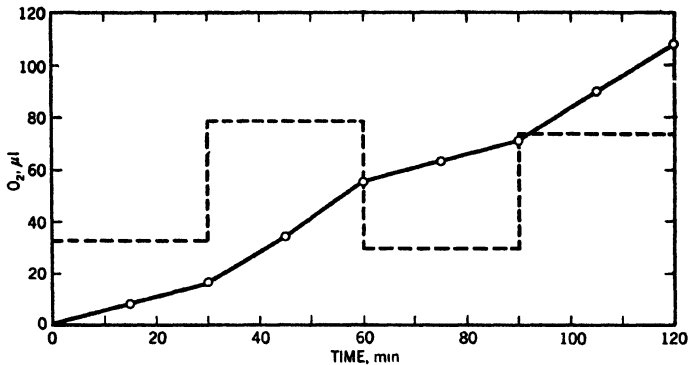


Fig. 5. Effect of carbon monoxide on respiration of sea urchin sperm (3 ml. suspension with  $1.3 \times 10^9$  sperm cells). Continuous line represents the oxygen uptake in an atmosphere of 90% carbon monoxide and 10% oxygen during alternating thirty minute periods of dark and light; broken line shows oxygen consumption rate per hour (252).

length. The nonreversal at 548  $m\mu$  is due to lack of absorption by cytochrome oxidase in this region of the spectrum. By interposing between the source of light and the microscope mirror a filter transmitting light of wavelength 548  $m\mu$ , Rothschild observed spermatozoa microscopically in the presence of carbon monoxide as if they were in the dark. Under such conditions he found that carbon monoxide reduces the respiration of spermatozoa without a corresponding reduction in motility. Robbie (249) reached a similar conclusion from his study of the effect of cyanide on sea urchin spermatozoa. Thus it is possible to create experimental conditions under which the respiration of sea urchin sperm can be dissociated from

motility. A further example of such behavior of spermatozoa was gained by Mann and Lutwak-Mann (200), who managed by using a suitable concentration of fluoride to abolish the motility as well as the fructolysis of ram sperm without suppressing completely the sperm respiration.

### C. OXIDIZABLE SUBSTRATES

In the presence of oxygen, ram or bull spermatozoa washed free from seminal plasma and thereby deprived of glycolyzable material still retain the ability to consume oxygen for an appreciable length of time, at a practically normal rate of respiration (152,188). This indicates that the aerobic metabolism of spermatozoa can be dissociated from fructolysis and is presumably the outcome of some endogenous processes involving direct combustion of intracellular material. According to Lardy and Phillips (142-144), the endogenous substrate utilized by bull sperm aerobically is not a carbohydrate at all, but a phospholipide; they are of the opinion that the motility of bovine epididymal spermatozoa is initiated and maintained by the oxidation of the phospholipide stores, with concomitant uptake of inorganic phosphate and the formation of an easily hydrolyzable ester, possibly identical with ATP. A similar process may underlie the respiration of ejaculated, washed spermatozoa. However, before the respiration of washed sperm suspensions can be declared a wholly endogenous process, proof must be offered first that the washing procedure has removed from ejaculated spermatozoa all extraneous material such as, *e.g.*, fructose. Past experience, however, shows how difficult it is to obtain washed spermatozoa absolutely free from fructose and fully motile at the same time. Moreover, if a calculation is made of the quantity of carbohydrate required to account for the entire oxygen uptake of washed sperm, the amount is found to be very small.

With regard to whole, ejaculated semen, there can be little doubt that the oxidation of carbohydrate predominates over other aerobic processes and constitutes an important source of metabolic energy for the spermatozoa. It also seems probable that, at least in those species which abound in seminal fructose, processes such as *e.g.*, the oxidation of phospholipides, come into prominence only after exhaustion of the fructose reserve and complete oxidation of the products

of aerobic fructolysis. This is borne out by measurements of the respiratory quotient of spermatozoa in the presence and in absence of glycolyzable substrates (95,116), by studies on the inhibitors of sperm respiration and glycolysis (147,148) as well as by some recent experiments of Mann and Lutwak-Mann (200) on the metabolism of dilute suspensions of washed sperm of bull and ram. The oxygen uptake of such suspensions was found to remain con-

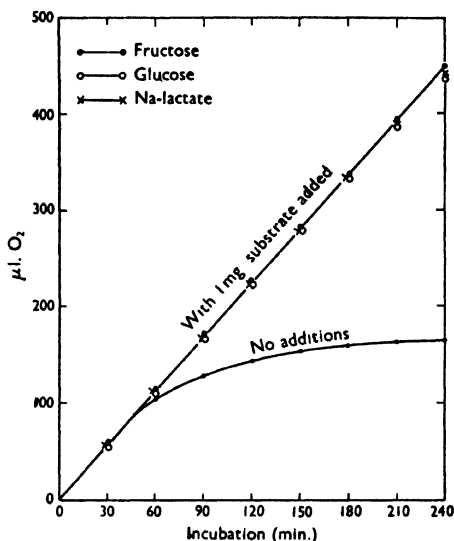


Fig. 6. Effect of carbohydrate on respiration of washed ram sperm (200). Oxygen uptake of 3 ml. suspension with  $0.45 \times 10^9$  sperm cells.

stant only for a limited period of time and to decline progressively unless an additional source of oxidizable material was provided in the form of fructose, glucose, or lactate. All these equally well maintained the initial rate of respiration for a considerable length of time, although none was capable of raising significantly the initial rate of oxygen consumption (Fig. 6). When added to respiring suspensions of washed ram sperm at a later stage, when the respiration had already begun to decline, these substances prevented further



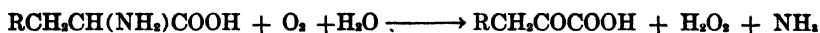
deterioration in the rate of oxygen consumption. The effect is not limited to fructose, glucose, and lactate, but is shared by several organic acids such as pyruvic, oxalacetic, propionic, butyric and acetic acid (111,112). Acetic acid has been also pointed out as a possible source of oxidation energy for bull spermatozoa (150). Succinic acid was found to be effective in the respiration of sea urchin spermatozoa (8,68), but it appears that the mode of action of the various substances which affect the respiration of sea urchin sperm differs fundamentally from the influence exerted by the same substances on mammalian spermatozoa. This is illustrated, for example, by the peculiar response of sea urchin sperm to such substances as malonate, iodoacetate, and nitrogen mustards, all of which were reported by Barron and colleagues (9,12) to have a marked stimulating effect on the sperm respiration.

#### D. HYDROGEN PEROXIDE FORMATION

Catalase was found in sea urchin semen (Lord Rothschild, 254). Slight catalase activity has also been detected in mammalian semen but it is debatable whether this activity is due to the sperm cells themselves or to some accidental contaminants of semen such as blood, pus, or bacteria (24,25,178,277). An extremely low level of catalase activity in normal and cleanly collected bull semen is so typical that Blom and Christensen (24,25) recommended it as a method for determining the "hygienic quality" of bull semen; the test employed in Denmark is performed in special "catalase tubes," in which the semen is treated with hydrogen peroxide and the volume of evolved oxygen measured. The above-mentioned observations deal with intact sperm cells. It is known, however, that there are enzymes such as, for instance, carbonic anhydrase in blood which differ in behavior when examined in intact erythrocytes as against plasmolyzed blood cells (130). On the other hand, even when ram spermatozoa were first homogenized, as described in Section II. B., and then the cell-free extract was tested for the presence of catalase, it took twenty minutes (at 18°C.) for an extract from 0.2 g. ram sperm (wet weight) to decompose a quantity of hydrogen peroxide split by 0.001 ml. blood in two minutes (196).

The lack of catalase in normal semen explains the toxicity of hydrogen peroxide toward spermatozoa as well as the harmful effect

of pure oxygen upon sperm motility. MacLeod (179) was first to point out that the toxicity of oxygen may possibly be due to hydrogen peroxide produced by the spermatozoa themselves in the course of their oxidative activity. This followed his observation that the addition of either hemoglobin or catalase to human semen prevented the spontaneous loss of motility in oxygen and also that hydrogen peroxide destroyed the sperm motility in a concentration as low as 1.5 to 3 parts per million. However, Tosic and Walton (294-297) chemically identified hydrogen peroxide as the product of certain aerobic processes in semen. Using "egg yolk phosphate pabulum," the semen diluent originally introduced into the practice of artificial insemination by Phillips and Lardy (230), Tosic and Walton found that the addition of egg yolk to bull sperm greatly increased the oxygen uptake, which, however, gradually declined in about one hour's time. On fractionation of the egg yolk they obtained an as yet unidentified nitrogenous substance, probably an amino acid derivative, which is readily oxidized by bull spermatozoa with the liberation of ammonia and hydrogen peroxide. The peroxide was identified by means of the benzidine peroxidase test, and conclusively proved to be the sole cause of the gradual decrease in the rate of oxygen uptake. Certain pure amino acids, such as L-tryptophan, L-phenylalanine and L-tyrosine, were found to be capable of replacing the nitrogenous component from egg yolk. According to Tosic (294), the hydrogen-peroxide-forming aerobic process in semen is an oxidative deamination catalyzed by L-amino acid oxidase in spermatozoa, according to the equation:



Some experiments by Evans (63) illuminated yet another aspect of peroxide formation when it was found that sea water heavily irradiated with X-rays, and used as a diluting medium for the sperm of *Arbacia punctulata*, led to serious reduction in the survival period of spermatozoa and also considerably delayed the first cleavage when these spermatozoa were used for fertilization of *Arbacia* eggs. The toxic agent in irradiated water has been identified as hydrogen peroxide and was successfully eliminated by the addition of catalase to the irradiated medium.

## VI. Citric Acid in Semen

### A. OCCURRENCE AND DISTRIBUTION

The occurrence of citric acid in semen was discovered by Schersten (265,266), who was also the first to point out that the acid is an extracellular component of semen and originates chiefly in the accessory organs of reproduction. Schersten's findings have since been confirmed and extended by several investigators (10,11,108,111, 112,151). In the reproductive organs of man citric acid is confined mainly to the prostate (10,11). In other mammals, however, such

TABLE III  
DISTRIBUTION OF FRUCTOSE AND CITRIC ACID IN MALE REPRODUCTIVE ORGANS

Organ	Weight of organ, mg.	Fructose, mg./100 g.	Citric acid mg./100 g.
Rabbit			
Glandula seminalis	120	21	50
Glandula vesicularis	820	75	180
Prostate	810	110	55
Cowper's gland	950	0	25
Rat			
Seminal vesicles	780	9	39
Coagulating glands	130	172	0
Median prostate	20	90	60
Ampullae	70	10	0
Dorsal prostate	250	82	20
Ventral prostate	320	0	122

as bull, ram, boar, and stallion, most of it is found in the seminal vesicle, i.e., the organ responsible for the formation of fructose (111, 112). In rabbit citric acid was found to be limited largely to the *glandula vesicularis*, whereas the highest concentration of fructose was found in the prostate (Fig. 7, Table III). In rat citric acid was located in the ventral prostate and the seminal vesicle proper, but fructose in the dorsal prostate and in the coagulating gland (Fig. 8, Table III).

### B. EFFECT OF MALE SEX HORMONE

Humphrey and Mann (111,112) have shown that there is a close relationship between the citric acid formation in the male reproductive system and the function of the testicular hormone. In this

respect citric acid behaves like seminal fructose, except, however, that at any rate in rabbit the postcastrate disappearance and the hormone-induced reappearance of citric acid in seminal plasma are not nearly as prompt as with fructose. It should be possible to adopt citric acid assay as an "indicator test" for the androgenic hormone, in a similar manner to fructose, as shown by the studies on castrated and hormone-treated bull calves (197) and rats (201).

Using subcutaneous transplants of seminal vesicle and coagulating gland tissue in rat, Mann, Lutwak-Mann, and Price (201) have

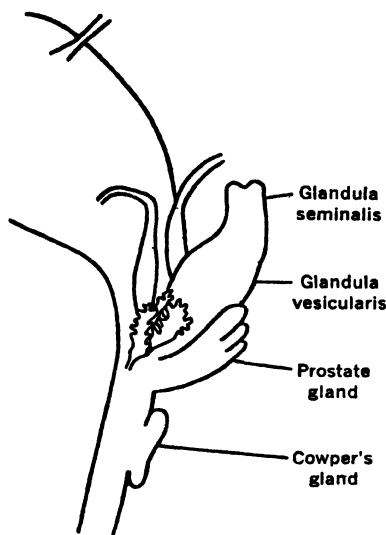


Fig. 7. Male reproductive organs of the rabbit.

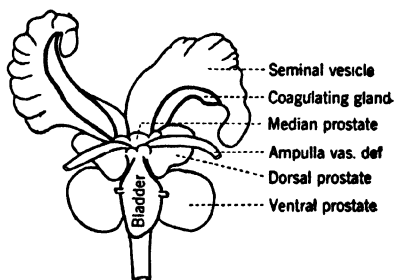


Fig. 8. Male reproductive organs of the rat (111).

shown that in transplants grown from coagulating gland plus seminal vesicle, both fructose and citric acid were present, whereas in those from coagulating gland alone fructose was found but citric acid was absent. In both instances, the production of fructose and citric acid strictly depended upon the male sex hormone. Another interesting example which illustrates the close relationship between androgens and citric acid was revealed during a recent study by Price, Lutwak-Mann, and Mann (240) of the so-called *female prostate* of rat. This organ, occasionally found in the female rat

and morphologically homologous to the male ventral prostate, was found to produce large quantities of citric acid in response to injections of testosterone.

### C. METABOLISM AND ROLE

When fresh semen is incubated *in vitro* citric acid is utilized by spermatozoa both aerobically and anaerobically. However, as compared to fructolysis, the rate of "citricolysis" is small and furthermore the two processes seem to bear no relation to each other, insofar as citric acid has no effect on the course of fructolysis in washed spermatozoa, and is incapable of maintaining sperm respiration (111,112). By washing the spermatozoa with Ringer solution, citric acid can easily be removed. It can be shown, however, that spermatozoa contain those enzymes which participate in the intermediary reactions of the "tricarboxylic acid cycle." This was first demonstrated by Lardy and Phillips (149,151), who also suggested that the "tricarboxylic acid cycle" may be involved in the normal sperm respiration.

As to the role, there has been a claim that citric acid exerts some beneficial effect on the sperm motility, suggesting that it may be specifically linked with sperm motility (151). The function of citric acid in semen remains, however, obscure, although it is conceivable that it may be connected with such phenomena as coagulation and liquefaction of semen (108), hyaluronidase activity (13), or the calcium-binding capacity of seminal plasma (102).

## VII. Sperm Survival and Its Dependence on Metabolic Processes

The discussion of this topic is difficult owing to the inadequacy of the available criteria for the appraisal of the fertilizing capacity of spermatozoa, and the survival period of sperm, which are frequently assessed in a semiquantitative manner by visual observations of the degree of sperm motility. Moreover, as Hammond (85) pointed out, even the correlation between motility and fertility is still somewhat uncertain and motile spermatozoa need not always be fertile.

Both the respiration and the anaerobic glycolysis of spermatozoa

have been suggested in the past as useful measures of motility and viability of mammalian spermatozoa. Measurement of respiration has been recommended by Walton (310,311) for practical assessment of the viability of bull semen specimens. Walton and Edwards (313) compared the breeding records of thirteen bulls, taking as a measure of their fertility the number of matings required to produce pregnancy in cows. They examined ten samples of semen from each of the thirteen bulls for sperm density and oxygen consumption, and found that there is some correlation between fertility and density, but an even closer one between fertility and the respiratory activity of the spermatozoa. A summary of their results is shown in Table IV. In the case of ram semen Chang and Walton (42) demonstrated a close relationship between respiratory activity and sperm motility.

TABLE IV  
CORRELATION BETWEEN FERTILITY AND RESPIRATORY ACTIVITY  
OF BULL SPERMATOZOA (310)

Number of bulls	Services per conception	Respiration, $\mu$ l. O <sub>2</sub> /10 <sup>6</sup> sperm/hr.	Total no. spermatozoa in ejaculate
3	1-1.9	0.058	1923 $\times 10^6$
6	2-2.9	0.038	1448 $\times 10^6$
4	3-6.9	0.012	1245 $\times 10^6$

Glycolysis was established as a useful method for assessing the quality of semen even before the seminal sugar was identified as fructose (46,140,179,215,259,317). However, the recent work on fructose and fructolysis brought out several new facts which help in the appraisal of semen quality and in the practical approach to several problems connected with male fertility (194,195,197,200). The fructose level in a fresh semen specimen depends upon the secretory function of the accessory glands of reproduction, particularly the seminal vesicles, closely integrated with the activity of the male sex hormone. Considerable fluctuations in the fructose content of semen are frequently noted even in the same individual. The causes of these variations are not clear at present; it is possible, however, that the fluctuations reflect periodic changes in the function of the male sex hormone, related perhaps directly to the capacity of the testes to produce testosterone, or else they may be the outcome of indirect influence of other hormones, *e.g.*, those of the

anterior pituitary, on the formation of testosterone in testes. A conspicuously low level of fructose in fresh semen, at least in some species, such as, *e.g.*, bull, is a pathognomic phenomenon and points toward some deficiency in the secretory function of the accessory genital apparatus. More often than not, it will be found associated with other symptoms of general hormonal dysfunction. It may also coincide with low quality of spermatozoa or necrosperry (194,195). It is worth recalling some earlier data of Anderson (3), who found that among 239 specimens of bull semen, 28 had a "reducing sugar" content of under 250 mg. per 100 ml. and that this abnormally low concentration of sugar usually went with a lowered rate of sugar disappearance during storage of the unbuffered semen. On the other hand, a high level of seminal fructose indicates good functional ability of the accessory glands; it does not, however, coincide necessarily with high density or motility of spermatozoa; azoospermic semen, for instance, as produced by vasectomized animals, usually has a very high fructose content, and semen of low density from subfertile animals can contain a normal amount of fructose (194,195). In this connection one may point to experiments by Moore and Mayer (215), who carried out a series of determinations of initial concentration of "reducing sugar" in ram semen and found that a high level of sugar coincided with high sexual drive.

In spite of the close relationship which exists between the content of fructose in semen and the male hormone activity in the body, great caution must be exercised in evaluating the hormonal activity from the results of fructose analysis, particularly in man, where the size and storage capacity of seminal vesicles, and consequently the amount of fructose in semen, are subject to exceptionally large individual variations. Harvey (89) in her recent survey of fructose in 150 specimens of human semen recorded values ranging from 5 to 640 milligram per cent; specimens with the minimum and maximum concentration alike came from fertile donors. Low values were found in specimens produced by donors after intervals of less than 24 hours, and usually, a period of 48 hours was required for fructose to reach the level characteristic of the individual donor. Thus it appears that a single ejaculation depletes the seminal vesicle to a much greater extent in man than in certain domestic animals; for instance, in the bull (194) we found the storage capacity of the seminal vesicles sufficient to yield eight ejaculates within one hour,

with a practically unaltered fructose content from first to last sample (see Table II).

The rate of fructolysis, as based on the assay of fructose disappearance, forms a simple and convenient yardstick of the metabolic activity of spermatozoa and compares very favorably with other biochemical methods, including the "methylene blue test" widely used at present for the appraisal of semen quality in the practice of artificial insemination (15,145,281,304). The normal rate of fructolysis in bull semen is 1.4 to 2 mg. fructose utilized per  $10^9$  sperm cells in one hour at  $37^{\circ}\text{C}$ . (194,195). Azoospermic and necrospermic semen is unable to utilize fructose; the rate of fructolysis is markedly reduced in sperm of low motility from subfertile and infertile animals. Addition of extra fructose at the start of the fructolysis assay to a semen sample which itself is rich in sugar has no marked effect on the metabolic performance of spermatozoa. An abnormally short period of sperm survival in spite of a plentiful supply of fructose in the seminal plasma can usually be accounted for by the presence in such semen of low quality spermatozoa. Occasionally, however, poor sperm survival is the outcome of an insufficient quantity and rapid exhaustion of fructose in semen; under such circumstances the addition of extra sugar, fructose (191) or glucose (262), may fulfill a useful purpose. It must be stressed, however, that the full effect can only be achieved if buffer is added simultaneously to counterbalance acidification, which exerts an injurious effect upon the sperm cells and thereby affects fructolysis (194,195). The maintenance of the fructose content greatly depends on temperature: in bull semen, at  $37^{\circ}\text{C}$ ., the disappearance of all fructose is a matter of a few hours; at  $5\text{--}10^{\circ}\text{C}$ ., which is the routine storage temperature for the purpose of artificial insemination, an appreciable proportion of bull seminal fructose remains intact even after several days.

A phenomenon of which we know little at present is the survival of spermatozoa in the female genital tract. In certain species, notably in some insects, the period of sperm survival in the female may extend over several years and presumably under such conditions the survival of spermatozoa depends on nutrients drawn largely from the secretions of the female tract. Another problem which awaits elucidation is the longevity of the sperm in the epididymis. No doubt the long survival of the spermatozoa in the epididymis is



partly due to their immotility. Here again, however, we are faced with the as yet unsolved question as to what constitutes the nutrient material for the epididymal spermatozoa.

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# NITROGEN METABOLISM OF HIGHER PLANTS

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## I. Introduction

Certain aspects of the nitrogen metabolism of higher plants were considered in detail by Chibnall (75) in 1939. Subsequently there have appeared a number of reviews, each considering in detail some more restricted aspect of this field (246,261,332,401). The present chapter, which is based primarily on work published since 1939, treats of the plant's nitrogen metabolism as a whole and directs

attention particularly to the enzyme systems involved and to the biochemical pathways of synthesis and degradation which they make possible.

## II. Assimilation of Ammonium and Nitrate Nitrogen

### A. EFFECT ON COMPOSITION OF THE PLANT

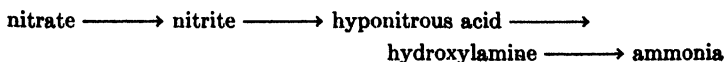
In most plants the ammonium ion is very rapidly metabolized and never accumulates in any appreciable amount in healthy plant tissues. Further ammonium nutrition has repeatedly been correlated with accumulation of amino acids and amides. Luxury supply of ammonium nitrogen leads to a high ratio of soluble organic nitrogen to total nitrogen and may even cause carbohydrate depletion and an associated increase in succulence (243). The ammonium ion apparently combines immediately with products of glycolysis, often notably with oxalacetic or  $\alpha$ -ketoglutaric acids with the formation of amino acids and usually of considerable quantities of one or both of the amides asparagine and glutamine. In a few plants characterized as "acid or ammonia plants" (197,283-285) ammonium nutrition or amino acid oxidation are associated with the accumulation of the ammonium salts of malic and oxalic acids. These plants, however, normally contain amides, though usually in low concentration, and there is no reason to suppose that their route of ammonium assimilation is fundamentally different from that in other plants. The synthesis of amide from ammonia and the corresponding keto acid would involve a reductive amination followed by the action of the specific amidase.

Nitrate nutrition is usually associated with pronounced accumulation of nitrate in the tissues and there is evidence that its rate of assimilation is often correlated with its concentration in the cells (243-245). Nitrate plants, in contrast to ammonium plants, usually have comparatively high contents of inorganic and of complex organic forms of nitrogen such as polypeptides (309,310,335). Luxury supply of nitrate does not lead to carbohydrate depletion; on the contrary, nitrate-fed plants have often been found to show carbohydrate accumulation as evidenced by accumulation of starch in the cells and increased woodiness of the tissues. Ammonium- and nitrate-fed plants are therefore clearly contrasted in their nitrogenous composition.

## B. MECHANISM OF NITRATE REDUCTION

Eckerson (110,112-114) has postulated that higher plants contain an enzyme, "reducase," responsible for the reduction of nitrate. Eckerson calculated the "reducase" activity from the rate of production of nitrite from nitrate in a mixture of expressed plant sap and glucose. The enzymic nature of the reducing system was, however, only inferred and both Anderson (8) and Loehwing (211) have claimed that the nitrate-reducing system is nonenzymic and dependent upon the presence of carbohydrate. More recently Bhagvat (39) has made a careful study of the aldehyde oxidase system of potato (36,231) and has shown that it catalyzes the oxidation of aldehydes by nitrate, that no coenzyme is necessary, and that it is destroyed by peroxide and inhibited by  $M/300$  potassium cyanide. Green, Strickland, and Tarr (145) have demonstrated the presence in bacteria of an enzyme system able to reduce nitrate to nitrite in the presence of a suitable oxidation-reduction indicator. The hypothesis that nitrate is reduced to nitrite in the plant is also supported by the evidence that, under suitable conditions, plants are able to utilize nitrite as a source of nitrogen and by the detection of nitrites in plants, particularly under conditions of rapid nitrate assimilation (110,116). The losses of nitrogen observed by Pearsall and Billimoria (258,259) when leaves of *Narcissus pseudo-narcissus* were supplied with nitrate under conditions causing accumulation of amino acids may be explained by postulating interaction of nitrite with amino acids. Vickery, Pucher, Wakeman, and Leavenworth (358) have also detected losses of nitrogen in developing leaves of *Narcissus poeticus* and attributed this to the formation of nitrite by oxidation of ammonia resulting from amino acid deamination.

The view that ammonia is the "alpha and omega" of the transformations of nitrogenous substances in the plant, developed from the pioneer researches of Schulze and Prianishnikov, suggested that the nitrate ion probably suffered reduction first to nitrite and then to ammonium and that ammonia was the compound concerned in the primary synthesis of organic nitrogen compounds. Chibnall (75) suggested that nitrate reduction probably involves the steps:



Corbet (83) has demonstrated that both hyponitrous acid and hydroxylamine are produced as intermediates in the oxidation of ammonia by nitrifying bacteria and Yamagata (408) has presented evidence that *Bacillus pyocyaneus* contains two enzymes responsible, respectively, for reduction of nitrate to nitrite and for further reduction of nitrite. Further, though both nitrite and hydroxylamine have been detected in higher plants supplied nitrate (202,203,232, 365), the experimental basis for the sequence of reactions postulated by Chibnall must be regarded as unsatisfactory.

The marked contrast in nitrogenous composition between plants receiving ammonium nitrogen as compared to those receiving nitrate nitrogen has led to the suggestion (309,310,313) that the actual course of protein synthesis may be somewhat different according to the nitrogen source utilized. This would be the case if nitrate is not reduced as far as ammonia before the nitrogen enters into organic combination. Virtanen (363), in work on the chemical mechanism of nitrogen fixation in leguminous plants inoculated with *Rhizobium*, obtained evidence that the atmospheric nitrogen is converted via unknown intermediates to hydroxylamine and that this then reacts with oxalacetic acid (373) to give an oxime which subsequently suffers reduction to L-aspartic acid. The isolation of oximinosuccinic acid from among the excretion products of the roots (371) strengthened this hypothesis. Virtanen and Arhimo (365) suggested that this sequence of reactions may be involved in nitrate assimilation and postulated that nitrate suffers reduction to hydroxylamine, that this then reacts with either oxalacetic acid or  $\alpha$ -ketoglutaric acid yielding the oximes, and that these are subsequently reduced yielding the respective dicarboxylic amino acids. The further stage of reduction of hydroxylamine to ammonia was then only likely to occur in the absence of the keto acids. The fact that only aspartic acid and not a mixture of aspartic and glutamic acids seemed to form the immediate end product of nitrogen fixation suggested the existence of a specific catalyst controlling the interaction of hydroxylamine and oxalacetic acid. However, no such enzyme is yet known and furthermore glutamic acid has now been detected (374) among the excretion products of inoculated pea roots. These new data, combined with the recent work of the Wisconsin school (60,396), have led Virtanen (364) to

accept considerable modification of his original hypothesis. Virtanen's present standpoint can be briefly summarized thus: Hydroxylamine is probably chiefly reduced to ammonia; oximes are formed through by-reactions, and the oxime of oxalacetic acid is formed in greater quantity than that of other keto compounds due to the greater rapidity of interaction of hydroxylamine with this acid. The detection of aminooxalacetic acid in the roots of inoculated pea plants has similarly led to the suggestion (338) that this may have arisen by interaction of nitrite with oxalacetic acid yielding the monoxime of diketosuccinic acid, which had then been reduced. Here again, however, it would seem that we are concerned with a by-reaction rather than a probable alternative path of nitrate assimilation. More recently Wood *et al.* (404a,b) have shown that both *Azotobacter* and oats can utilize oximinosuccinic and  $\alpha$ -oximinoglutaric acids as nitrogen sources, although it is not possible to establish by analysis of the organisms that the oximes are absorbed unchanged. The amount of protein formed in the oat seedlings grown in solutions containing these oximinodicarboxy acids in some cases approached that of controls in nitrate solutions of the same molarity. It is suggested that oximinodicarboxy acids may be intermediates in the synthesis of amino acids from  $\alpha$ -keto acids when nitrogen is supplied as nitrate. At present, however, this evidence for an alternative route of nitrate assimilation fails to explain the marked contrast in nitrogen biochemistry between nitrate- and ammonium-fed plants.

Baudish (24) carried out a series of *in vitro* experiments from the results of which he was led to suggest that the first step in nitrogen assimilation by plants was the photochemical production of formhydroxamic acid,  $\text{HC(OH)=NOH}$ , by interaction of nitrite with activated formaldehyde, and that the formhydroxamic acid then reacted with additional activated formaldehyde to form complex nitrogenous substances. Baly developed this theory further (20). The biosynthesis of amino acids by photochemical reactions between nitrates and substances such as glycol and glucose was postulated by Dhar and Mukerjee (102). However, no impressive body of evidence has ever been advanced in support of these theories. Evidence that light is essential or at least has a marked stimulating effect on nitrate assimilation has been obtained by many workers

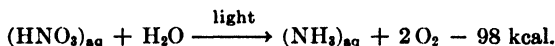
(40,96,104,114,135,201,259,312) and frequently an accumulation of nitrates has been recorded under short day, as compared with long day, conditions (157,162,248). It is, however, equally clear from the literature reviewed by Nightingale (243,245a) that in some plants active nitrate assimilation occurs in the root, and it has been possible to detect both nitrite and hydroxylamine in the roots of peas and oats supplied nitrates and grown in either light or darkness (365).

Burström (66) has suggested that earlier and apparently contradictory evidence regarding the role of light in nitrate reduction can be explained if it is accepted that nitrate assimilation can take place in both roots and leaves but that, while in the former the energy for the endothermal reactions involved is supplied by the breakdown of carbohydrate, in the leaves the nitrate reduction is linked with photosynthesis and is dependent upon both light and carbon dioxide. In the roots the products of nitrate reduction are visualized as combining with intermediates of carbohydrate breakdown. Evidence has been obtained (61-65,250) that manganese is essential for nitrate reduction in the root. Burström has suggested that a single oxidation-reduction reaction accomplishes the whole reduction of nitrate to ammonium and that the influence of pH on the rate of nitrate assimilation is explicable in terms of the effect of pH on Mn absorption. Burström visualizes nitrate assimilation in the leaf as being effected by combination of intermediates in carbohydrate synthesis with a light-activated derivative of nitrate or with nitrate itself. Here, the rate of nitrate assimilation is related to both light intensity and carbon dioxide concentration, and the increased carbon dioxide assimilation in the nitrate-containing as compared to the nitrate-free cultures is not associated with increased carbohydrate formation but with the formation of what Burström termed "CN assimilates." The much earlier work of Warburg and Negelein (386) on nitrate assimilation by *Chlorella pyrenoidosa* can be interpreted as supporting such a theory of a photochemical reduction of nitrate in green plants exposed to light (273). The equation for the dark reduction of nitrate can be represented thus:



A marked stimulation of carbon dioxide evolution due to this "nitrate respiration" was recorded. In light, this evolution of carbon

dioxide due to "nitrate respiration" was replaced by the evolution of oxygen in greater volume than that of the carbon dioxide assimilated. The replacement of carbon dioxide evolution by an equivalent evolution of oxygen would be explicable by utilization of the carbon dioxide in photosynthesis. The further stimulation of oxygen evolution requires the postulation either that the "nitrate respiration" is enhanced in light by increased nitrate absorption (the view adopted by Warburg and Negelein) or that nitrate is also reduced in light by photochemical reactions involving either an organic reductant or water as hydrogen donor. The photochemical reduction of nitrate by water can be represented thus:



A similar stimulation of oxygen evolution resulting from enhanced nitrate supply was also observed in *Elodea* (213). Tottingham and Lease (344) have drawn attention to the stimulating effect of short wave visible light and of radiations in the near ultraviolet on nitrate reduction in wheat and tomato, but the bearing of this work on the hypothesis of a photochemical reduction of nitrate is obscure.

Eckerson (110-114) concluded that potassium, calcium, phosphorus, and sulfur were all essential for nitrate reduction but the evidence remains inconclusive; recent work indicates that deficiencies of these elements interrupt nitrogen metabolism by their effect on nitrate absorption rather than on its reduction (14,54,55,59,88,134,152,200,218,244,279,342). Wall (384) has also brought forward evidence that potassium, after absorption, is concerned with amino acid condensation rather than with nitrate reduction and Wall and Tiedjens (385) and Richards (277) have obtained evidence that potassium is equally important for the transformation of the ammonium ion into soluble organic nitrogen and protein. Beckenbach, Robbins, and Shive (26) have concluded that the effect of potassium on nitrogen metabolism is indirect and is dependent upon the importance of potassium in the release of energy in the tissues, a hypothesis which does not conflict with the observations of Steward and Preston (329) that potassium stimulates respiration and protein synthesis in potato tuber tissue. Eaton (107) considered that his data supported the view that sulfur deficiency resulted in low



"reducase" activity; but here again Petrie (261) has pointed out that there was hardly any accumulation of nitrate in the sulfur-deficient as compared to the normal plants. Though manganese may be involved in nitrate assimilation by the root (61-65,250), Arnon (13) has emphasized the importance of manganese supply to plants supplied ammonium nitrogen; Vlasyuk (377) has obtained results suggesting that manganese is equally beneficial to both nitrate- and ammonium-fed plants, through its general importance in maintaining oxidation-reduction balance in the tissues. Though Steinberg (320,321) was able to show that molybdenum was essential for *Lemna* supplied with nitrate nitrogen, no corresponding ammonium series was carried out. Furthermore, the work of Arnon (13) and Arnon and Stout (16) indicated that molybdenum was essential for ammonium-fed plants. More recently, however, Mulder (239), in experiments using tomato and barley, has produced strong evidence that molybdenum is essential for nitrate, as contrasted with ammonium, assimilation. This author concludes that, although it is possible that molybdenum is required for some other reaction in the life of the plant, one of its main functions is that of catalyzing nitrate reduction. Nevertheless, the evidence in favor of the essential nature of most of these elements for the reactions involved in nitrate reduction is unsatisfactory and, as Blackman and Templeman (40) have observed, it seems highly improbable that all these factors are directly concerned in the formation or activation of the simple enzyme chain presumably involved.

The need for further research into the mechanism of nitrate assimilation in the higher green plant and into the cause of the contrasted nitrogen metabolism of nitrate and ammonium plants is very evident.

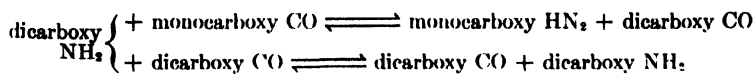
### III. Synthesis and Degradation of Amino Acids

#### A. AMINO GROUP INTERCHANGE

An assessment of our present knowledge of this aspect of plant metabolism is only possible if it is considered in relation to parallel studies on the metabolism of amino acids in animal cells. Schoenheimer and co-workers (293) have used heavy nitrogen (the isotope  $^{15}\text{N}$ ) as a marker of the amino, imino, and other nitrogenous groups

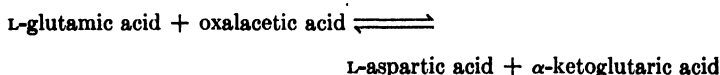
and have shown that, whenever isotopic amino acids or ammonia (the compound used contained a higher percentage of  $^{15}\text{N}$  than the naturally occurring compound) are fed to animals and the subsequently isolated body proteins analyzed, the isotope concentration in the dicarboxylic amino acids is much higher than in the other amino acids (with the exception of the one in which the isotope was administered) and that the concentration of isotope in glutamic acid is always somewhat higher than in aspartic acid. Vickery *et al.* (355) have supplied isotopic ammonium chloride to tobacco plants over a period of three days, and again, as in the animal experiments, subsequent hydrolysis of the tissue proteins showed that the glutamic and aspartic acids contained a significantly higher proportion of the isotope than that found in the other amino acids. Hevesy *et al.* (156) carried out a parallel experiment at Carlsberg using sunflower and obtained similar results. This work indicates that the dicarboxylic amino acids probably play some special role in the nitrogen metabolism of both plants and animals. Other experiments using  $^{15}\text{N}$  have emphasized that there is a constant interchange of nitrogen-containing groups between the body proteins and the simpler organic nitrogen compounds, and that within the latter fraction rapid exchange of amino nitrogen is continuously proceeding. These researches seem to require for their explanation the existence in both plant and animal cells of some mechanism for the rapid interchange of amino groups and involving the dicarboxylic amino acids as key substances.

It is necessary, therefore, to examine how far the transamination mechanism ("unaminierung") first described by Braunstein and Kritzman (52) is adequate to explain the results obtained with heavy nitrogen. Braunstein and Kritzman (52,53,194,195) consider that two enzymes, a glutamic and an aspartic aminophorase, occur, that the transamination mechanism allows a general interconversion of amino acids, and that the dicarboxylic amino acids or their corresponding  $\alpha$ -keto acids together with the appropriate enzyme are the essential components of the system. The two systems are represented thus:

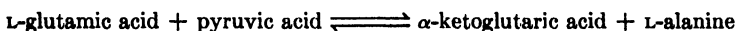


Amino group transfer takes place only through the intermediary of either a dicarboxylic amino or keto acid, although the dicarboxy compounds are required only in catalytic amounts. Braunstein and Kritzman claim to have demonstrated that sixteen different natural and racemic amino acids were able to donate amino nitrogen to  $\alpha$ -ketoglutaric acid in the presence of the glutamic aminophorase of animal tissues. Kritzman (195) reported that plants are rich in the aspartic aminophorase but appear to lack the glutamic enzyme. von Euler *et al.* (119), however, had earlier reported that various plants contain an enzyme system catalyzing amino nitrogen transfer in presence of glutamic acid or  $\alpha$ -ketoglutaric acid, and Virtanen and Laine (370,372) had obtained evidence for the existence in crushed pea plants of both aminophorases.

Cohen (77) has subjected the work of Braunstein and Kritzman to criticism and concluded that significant transamination in animal tissues takes place only with alanine, glutamic and aspartic acids, and their corresponding keto acids and that all other amino acids tested show either very low or no activity. Further, Cohen (78,80) has advanced the view that only one "transaminase" enzyme is involved and that the only reaction which proceeds with rapidity is:



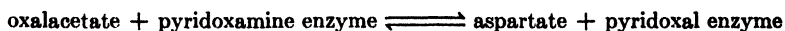
The reaction:



was found to proceed at about one-fifth the rate, and the reaction of aspartic with pyruvic acid only to an inappreciable extent. Cohen (79) has compared the transamination activity of various tissues and found that the seeds and seedlings in which transaminating systems have been reported show an extremely low rate of amino group transfer as compared to animal tissues. From this he concluded that either transamination plays a still less significant role in plants than in animals or that the methods of extracting and purifying the enzyme preparations cause considerable inactivation when applied to plant tissues. Cedrangolo and Carandante (71) have, however, reported that aqueous extracts of various seeds and seedlings are very active in transaminating the system  $\alpha$ -ketoglutaric acid + aspartic acid and more recently Rautanen (274), in experi-

ments with green plants, has obtained evidence which, while supporting the restricted nature of the transaminase system, indicates that the reactions of aspartic with  $\alpha$ -ketoglutaric acid and of  $\alpha$ -ketoglutarate with alanine both proceed at an appreciable rate. The aromatic amino acids and their corresponding keto acids, however, showed no activity. The difficulty of interpreting results in this field is emphasized by the work of Lichstein and Cohen (205) who, in a study of transamination in bacteria, have reported the presence of an active transaminase system catalyzing the reaction of glutamic with oxalacetic acid and have interpreted the negative results of Diczfalusy (103) as due to the latter's attempt to use as a measure of transaminase activity the reverse reaction of aspartic with  $\alpha$ -ketoglutaric acid or the reaction of  $\alpha$ -ketoglutaric acid with alanine, both of which proceed very slowly.

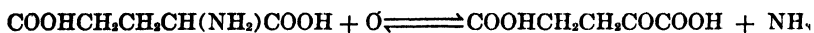
Albaum and Cohen (3) have studied transamination in germinating oat seedlings. The interaction of glutamic with oxalacetic acid proceeded at a very rapid rate, the reverse reaction at about one-third the forward rate. The transaminase activity was found to increase ahead of the rising rate of protein synthesis and there appeared to be a close relationship between transaminase activity and protein synthesis. It was suggested that transamination is of significance in protein synthesis in a manner quite distinct from the interconversion of amino acids and that it can effect such interconversion only to a very limited extent. Recent work (143,206, 290,291) has shown that transaminases from various sources are associated with pyridoxal phosphate as the prosthetic group, and Snell (315) has suggested that the process of transamination should be represented:



Transaminase systems appear to be of widespread occurrence but the recent work here considered seems to cast serious doubt on their significance in the general and separate synthesis of amino acids, particularly of the aromatic amino acids (154). It would seem probable that mechanisms additional to transamination are involved in the widespread amino group transfer revealed by work with  $^{15}\text{N}$ .

### B. $\alpha$ -KETO ACIDS AS AMINO ACID PRECURSORS

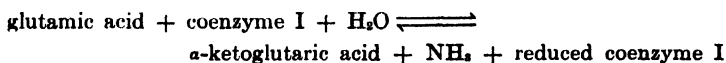
The concept of amino acid synthesis envisaged by many workers (75) postulates the  $\alpha$ -keto acids as their immediate precursors. The conversion of these acids to their corresponding amino acids is considered to take place either by transamination or by a reductive amination. The previous discussion has emphasized that there is considerable doubt regarding the general significance of transamination particularly in plant cells. Furthermore it could only serve to effect the transfer of existing amino groups and would not account for their primary synthesis. The process of reductive amination is visualized as the reverse of oxidative deamination. The L-glutamic dehydrogenase of animal tissues, which oxidatively deaminates glutamic acid to  $\alpha$ -ketoglutaric acid, has been subjected to detailed study by von Euler *et al.* (119) and Dewan (101). The reactions involved can be represented thus:



The intermediate formation of the corresponding imino acid:



is postulated. Since coenzyme I (DPN) acts as coenzyme of the system, the reaction can be written thus:



Similar enzymes from yeast (118) and from higher plants (28, 92, 120) have been described. Krebs (186) has also obtained evidence for the presence in liver and kidney tissue of a separate enzyme system not specific for glutamic acid but able to oxidize a number of natural amino acids to their corresponding keto acids and ammonia. This enzyme system, termed L-amino acid oxidase, however, could not at the time be obtained free from cell debris without loss of activity, though other data indicated it to be a flavoprotein. More recently, the enzyme has been submitted to intensive study (42, 43, 144) and obtained in a highly active state free from cell debris and electrophoretically homogeneous (233). It has proved to be a flavoprotein with riboflavin phosphate as the prosthetic group. This L-amino acid oxidase is able to catalyze the oxidation of thirteen natural amino acids, but is inactive with glycine, threonine, serine, glutamic and aspartic acids, lysine, ornithine, and arginine.

*In vitro*, both the L-glutamic hydrogenase and the L-amino acid oxidase effect oxidative deamination. It is necessary therefore to consider the evidence that their reactions can be reversed *in vivo*. Using tissue slices, it has been possible to demonstrate the synthesis of alanine from ammonium pyruvate by liver tissue (188) and of glutamic acid from  $\alpha$ -ketoglutaric acid by brain tissue (390). Perfusion with other  $\alpha$ -keto acids has, however, given negative results (188). The only substantiated amino acid syntheses from  $\alpha$ -keto acids therefore involve acids known to be active in the transamination reaction.

Chibnall (75) has demonstrated that when blades of perennial rye grass are infiltrated with the ammonium salt of  $\alpha$ -ketoglutaric acid, glutamine is synthesized to an amount corresponding to the  $\alpha$ -ketoglutaric acid disappearing from the tissues. This may, however, be due to a combination of transaminase and glutaminase activity. The conception that in plants amino acids are synthesized from  $\alpha$ -keto acids does not rest on any large body of evidence. The only  $\alpha$ -keto acids known to occur in higher plants are  $\alpha$ -ketoglutaric acid, oxalacetic acid, and pyruvic acid. The oxidative deamination of amino acids, other than glutamic acid, has not been extensively studied in plants. L-Amino acid oxidase has not been identified as a general plant enzyme and Boswell (51), in a study of the oxidation systems in the potato tuber, has concluded that the polyphenol oxidase, in association with the natural polyphenol present in the cells, is responsible for the oxidation of amino acids and that these are the normal hydrogen donors reducing the quinone compound. This work is in agreement with the hypothesis advanced by Steward and Preston (328) to explain the interrelationships between nitrogen metabolism and respiration. It is interesting to note that Boswell emphasizes that his work relates to the mechanism of the oxidative deamination and that the mechanism by which amino acids are synthesized within the tissue awaits further investigation.

### C. GLUTAMIC ACID AS PRECURSOR OF OTHER AMINO ACIDS

Even if it is regarded as probable that the dicarboxylic amino acids and alanine are synthesized in plant cells directly from the products of glycolysis, most likely the corresponding  $\alpha$ -keto acids, the route of synthesis of other amino acids remains obscure. These



and Rittenberg (307), from studies of the metabolism of  $^{15}\text{N}$  fed to rats as isotopic glycine, have obtained evidence which would fit in with the existence of a cycle of transformation from proline via  $\alpha$ -keto- $\delta$ -aminovaleric acid, ornithine, and  $\alpha$ -amino- $\delta$ -iminovaleic acid to glutamic semialdehyde and from glutamic semialdehyde either to glutamic acid or via pyrrolinecarboxylic acid back to proline (Scheme I).

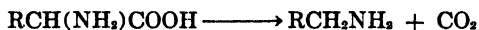
Kiesel (175) demonstrated that wheat seedlings can convert arginine into ornithine, and later (176), using *Vicia sativa*, demonstrated the presence of the enzymes arginase and urease. Citrulline has been isolated from melon juice (379). The existence in higher plants of all the components involved in the ornithine cycle (185, 192) suggests that arginine may be formed via ornithine from glutamic acid. It is possible therefore that glutamic acid functions, by biochemical pathways distinct from transamination, as the natural precursor in plants of several other amino acids.

The recent work with biochemical mutants of *Neurospora* and *Escherichia* opens up new possibilities for investigating the routes of synthesis of amino acids. Bonner, Tatum, and Beadle (48) have obtained a strain of *Neurospora* requiring isoleucine and valine, and a study of its behavior showed the existence of a close biochemical relationship between these two amino acids and leucine. A mixture of the  $\alpha$ -keto acids corresponding to isoleucine and valine was inactive, but either keto acid fed with the other amino acid was active in promoting growth. Beadle and Tatum (25) have described two tryptophan-less mutants of *Neurospora* one of which will grow in the presence of indole and the other in presence of either indole or anthranilic acid. Later it was shown (341) that indolepropionic, indolelactic, or indolepyruvic acids were, among other compounds, inactive and that the evidence pointed to serine and indole as the immediate precursors of tryptophan. Doermann (105) has studied a lysine-less mutant of *Neurospora* and shown that its growth is not only dependent upon a supply of lysine but is markedly inhibited by L-arginine. This led to the suggestion that the normal strain, not inhibited by arginine, may in fact not carry out a primary synthesis of lysine but synthesize lysyl peptides from the corresponding aldehydes (173,207) (Sch. II). The mutant strain would then be regarded as unable to synthesize the aldehyde and therefore must form the essential peptides directly from lysine,





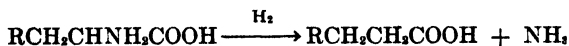
sibly also of synthesis) which have been investigated to any extent are those of oxidative deamination and transamination. A much greater diversity in the pathways of amino acid degradation is established from studies of the nitrogen metabolism of bacteria (7). It is, therefore, pertinent to inquire whether any evidence at all exists for the occurrence of such alternative pathways in the cells of higher plants. The production of amines from certain amino acids by bacterial putrefaction has long been known (21,281). Gale (131,132) has shown that these amines are produced by the action of specific amino decarboxylases of which six have been characterized, namely, those attacking the amino acids L-lysine, L-ornithine, L-arginine, L-tyrosine, L-histidine, and L-glutamic acid. There is also indirect evidence for the existence of an L-aspartic acid decarboxylase. The general reaction involved in amino acid decarboxylation is:



Okunuki (252) obtained evidence of the existence in a number of higher plants of a specific glutamic acid decarboxylase but was unable to obtain the enzyme in cell-free solution. More recently, Schales *et al.* (287,288) have obtained this enzyme in clear solution, studied its properties, and obtained evidence that it is a pyridoxal phosphate-protein complex. It appears to be widely distributed and some decarboxylase activity was found in all of the 34 species of plants examined. The metabolic significance of the conversion of glutamic acid into  $\gamma$ -aminobutyric acid is obscure, unless this route of amino acid breakdown can under certain conditions serve as a valuable source of carbon dioxide. It has been shown that (369,375) *Rhizobium leguminosarum* can form  $\beta$ -alanine from aspartic acid, and therefore apparently contains an aspartic decarboxylase. Pantothenic acid, which is widely distributed in higher plants, has  $\beta$ -alanine as a constituent part of its molecule. Green plants can, when photosynthesis is taking place, synthesize pantothenic acid (216) and it is therefore possible that, for this synthesis,  $\beta$ -alanine is obtained by decarboxylation of aspartic acid. A number of alkylamines are widely distributed in plant tissues (149) and of these methylamine could arise from glycine by decarboxylation, isobutylamine from L-valine, and isoamylamine from L-leucine. Amino acid decarboxylases active against these monoaminocarboxylic acids are,

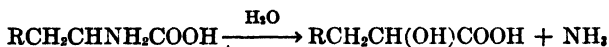
however, as yet unknown and the significance of amino acid decarboxylation in higher plants remains obscure.

Microorganisms are also able to effect amino acid degradation either by reduction or hydrolysis. The process of reductive deamination can be represented thus:



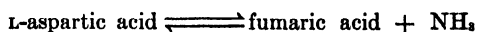
This route of degradation is confined to bacteria, yeasts, and molds and occurs only under anaerobic conditions. Strickland (336,337) found that certain *Clostridia* under anaerobic conditions coupled reductive deamination of glycine, proline, or hydroxyproline with simultaneous oxidative deamination of other amino acids.

The hydrolysis of amino acids can be represented thus:



This reaction, effected by certain bacteria, yields as the immediate decomposition products  $\alpha$ -hydroxy acids. By this general reaction glycine would yield glycollic acid, and aspartic acid would yield malic acid. These and other hydroxy acids occur in higher plants but there is no evidence that they represent the immediate decomposition products of amino acids.

The coli-typhoid group of bacteria are able to attack the  $\alpha,\beta$  linkage of amino acids liberating ammonia and forming substituted acrylic acids. By this mechanism aspartic acid is converted to fumaric acid, and histidine to urocanic acid. Quastel and Woolf (272) described the presence of an enzyme aspartase present in *Escherichia coli* which catalyzed the reaction:



Furthermore under anaerobic conditions L-aspartic acid was synthesized from ammonium fumarate. The same enzyme isolated from *Bacillus fluorescens liquefaciens* (376) was shown to be highly specific, only capable of effecting the deamination of aspartic acid and catalyzing uptake of ammonia with fumaric acid, but not in presence of other closely related acids (mesaconic and trans-aconitic acids). Weak aspartase activity was also found (376) in pea seedlings and in leaves of young red clover. Later, Ishihara (165) reported aspartase to be widely distributed in higher plants. The demonstration of the occurrence in higher plants of a succinic

oxidase (93), which converts succinic acid into fumaric acid, and the considerable evidence indicating that succinic acid can act as a nonnitrogenous precursor of asparagine (146,237) suggest that aspartase is involved in one chain of reactions whereby asparagine is synthesized. The suggested origin of asparagine from malic acid in beet tissue under anaerobic conditions would also, presumably, involve the enzymes fumarase and aspartase (241). The significance in higher plants of the enzyme aspartase in aspartic acid synthesis and degradation, therefore, rests on experimental evidence. Stephenson (322) suggests that, in bacteria, the formation of substituted acrylic acids from amino acids may be a general route of degradation. It is, however, not yet known how far specific enzymes are involved for each amino acid, nor have the unsaturated acids corresponding to any number of amino acids been identified as products of bacterial metabolism. It may be noted in this connection that the plant acids cinnamic acid and *p*-coumaric acid are the unsaturated acids corresponding to phenylalanine and tyrosine, respectively. The significance of this general reaction in the metabolism of higher plants is, however, with the exception of the aspartase system, purely speculative.

The above discussion serves to emphasize our lack of detailed knowledge regarding the biochemical pathways leading to amino acid accumulation and utilization in higher plants. This represents a serious handicap in the consideration of theories relating to the mechanism of protein synthesis. Only in the case of glutamic and aspartic acids and of alanine is it possible to trace routes of synthesis and degradation. Furthermore, from our knowledge of the metabolism of microorganisms and higher animals and from such data as are available from biochemical investigations of higher plants, it would seem that glutamic acid and to a lesser extent aspartic acid occupy a central position in the biochemistry of amino nitrogen.

#### IV. Role of Acid Amides

The early discovery of asparagine and glutamine, the early development of methods of estimating amide nitrogen, and the marked accumulation of amides, particularly asparagine, in certain etiolated seedlings first focused attention on the role of acid amides. The pioneer researches of E. Schulze (1872-1898) and of D. N.

Prianishnikov (1895-1922) based our early knowledge of the nitrogen metabolism of higher plants upon work directed at the elucidation of the role of acid amides and particularly asparagine. The history of their discovery has been reviewed by Vickery *et al.* (356,359) and the historical development of our knowledge of amide metabolism has been traced in detail by a number of workers (75, 238,240,331). Subsequently, Archibald (12) has given us a very full summary of the chemical properties of glutamine and of its role in animals and plants. The present discussion is therefore mainly confined to a survey of recent work and gives particular consideration to the evidence indicating similarities and difference in the metabolism of glutamine as compared to asparagine.

#### A. AMIDE ACCUMULATION

Amide accumulation occurs most markedly either in response to feeding with ammonium salts or during the catabolic changes which occur in etiolated seedlings or detached leaves. Protein breakdown under anaerobic conditions leads to amino acid accumulation (68, 69,255,339,387,404) but under aerobic conditions amino acids suffer oxidation and, while carbohydrate reserves are available, amides accumulate. As carbohydrates are depleted amide formation is checked (270) and eventually the final stages of catabolism are marked by ammonia accumulation. Reversal of catabolism achieved by exposure to light or by direct augmentation of the sugar supply results in utilization of the amide and amino nitrogen for resynthesis of protein (266,314,383).

It has been shown (90,91) that asparagine and glutamine occur as such in the protein molecule and therefore will arise during enzymic hydrolysis in the absence of amidases. It is clear, however, that the amounts of the amides accumulating in etiolated seedlings are far greater than could arise from the dicarboxylic amino acid content of the reserve protein broken down (75,297,298,300,305) so that the greater part of the amide nitrogen must have arisen by combination of ammonia (liberated by deamination of amino acids) with either nitrogen-free precursors or with dicarboxylic amino acids secondarily derived from other amino acids.

Many species of plants growing under otherwise normal conditions but supplied with nitrogen solely and in luxury amount as ammonium salts accumulate amides. Furthermore, in ammonium-

fed plants, the sum of the amide and amino nitrogen of the accumulated amides has been found to correspond closely to the total ammonium absorbed (271,351) suggesting that amide frequently arises by combination of ammonia with nitrogen-free precursors. Chibnall (75), for instance, has shown that blades of perennial rye grass infiltrated with the ammonium salt of  $\alpha$ -ketoglutaric acid produce glutamine and that the whole of the metabolized ammonia is almost exactly accounted for by the sum of the amino and amide nitrogen of the glutamine synthesized.

### B. PRECURSORS OF AMIDES

These general trends in amide metabolism suggest that asparagine and glutamine can under suitable conditions serve as readily available neutral sources of nitrogen for protein synthesis and that they arise by combination of ammonia (either supplied as such or resulting from protein breakdown) with nitrogen-free carbon skeletons often derived directly from the products of glycolysis. Chibnall (75) has considered in detail biochemical pathways which may connect such possible immediate amide precursors as oxalacetic,  $\alpha$ -ketoglutaric, and fumaric acids with the end products of sugar glycolysis and fat metabolism; both Chibnall (75) and Vickery *et al.* (349, 356) have tentatively postulated that a citric acid cycle (193) may be operative in plant tissues. Through such a cycle, malic, succinic, and citric acids could yield the immediate precursors of the amides. Mothes (237) has in fact recorded asparagine synthesis in response to the infiltration of leaves with the ammonium salts of malic and succinic acids. These results have, however, been criticized by Schwab (304), who claims that amide synthesis occurs equally well whether the acid supplied is malic or sulfuric acid. Similarly Chibnall (75), who infiltrated leaves of perennial rye grass with the ammonium salts of pyruvic, glutaric, and glutaconic acids, was unable to demonstrate that the acid ions had been utilized in glutamine synthesis, and in fact obtained pronounced amide synthesis in response to infiltration with ammonium hydrogen phosphate. Vickery *et al.* (356) were also unable to correlate glutamine or asparagine synthesis with utilization of either citric or malic acid in tobacco leaves. Furthermore James (167) has recently drawn attention to evidence against the importance of an organic acid cycle in plant respiration. The hypothesis that an organic acid cycle

is operated in plants and is of importance in amide formation must therefore be accepted with caution. The lack of correlation between citric acid metabolism and glutamine would be explained if, as is now suggested (121,398) *cis*-aconitic acid is the intermediate between oxalacetic and  $\alpha$ -ketoglutaric acids, and citric acid is interpreted as the end product of a side reaction. Lack of knowledge of the mechanism whereby oxalacetic acid arose from pyruvic acid represented a grave theoretical weakness in the organic acid cycle until it was shown that molds and bacteria can utilize carbon dioxide as a metabolite and that this carbon dioxide was, at least in part, used for the carboxylation of pyruvic acid (191,392). Ruben and Kamen (282) have shown that unicellular algae also effect the carboxylation of pyruvic acid by carbon dioxide to give oxalacetic acid, and Bonner and Bonner (49) have obtained evidence that in *Bryophyllum* and *Mesembryanthemum* there takes place a direct fixation of carbon dioxide as organic acid probably by the Wood and Werkman (392) reaction. It is also to be borne in mind that the occurrence in plant tissues of citric, isocitric, malic, fumaric, succinic,  $\alpha$ -ketoglutaric, oxalacetic, and pyruvic acids is almost certain and that a number of the enzymes involved in the citric acid cycle have now been demonstrated to occur in plants: succinic oxidase (93), malic dehydrogenase (9,161,381), aconitases (166,225), isocitric dehydrogenase (28,120). Papers by Berger and Avery (28), Machlis (222), and Henderson and Stauffer (153) all present evidence in favor of the functioning of an organic acid cycle in plant respiration.

### C. SOURCE OF ENERGY FOR AMIDE SYNTHESIS

The aerobic conditions required for amide synthesis would appear to be of importance not only for effecting oxidative deamination of amino acids but also for the release from protein, carbohydrate, or other substrate of respiratory energy. Krebs (187) has shown that in kidney cortex and in brain tissue the synthesis of glutamine is linked with aerobic respiration and that hydrocyanic acid inhibits the synthesis of glutamine to approximately the same degree as it inhibits respiration. Under anaerobic conditions, no glutamine synthesis takes place in the tissues and in the presence of the specific enzyme concerned glutamine is, *in vitro*, completely hydrolyzed to glutamic acid and ammonia. More recently, Speck (316) and Elliott

(117) have shown that adenosine triphosphate is the immediate energy donor for glutamine synthesis and Elliott has suggested that the first stage in this synthesis may be a transfer of a phosphate group of adenosine triphosphate to the  $\gamma$ -carboxyl group of glutamic acid forming glutamyl phosphate. This could then be expected to react with ammonia to form glutamine with the liberation of inorganic phosphate. McRary (221) has pointed out that the conversion of ammonium aspartate to asparagine at pH 7.0 involves a standard free energy of synthesis of 3460 cal. and concluded that in his seedling cultures energy supply was a limiting factor in asparagine synthesis under conditions of adequate nitrogen supply.

#### D. RELATIVE ROLES OF ASPARAGINE AND GLUTAMINE

##### 1. Contrast between Different Plant Species

The early work of Schulze and of Prianishnikov on the amide metabolism of seedlings was directed to a study of asparagine formation and utilization. Schulze, however, drew attention to the fact that glutamine, rather than asparagine, accumulated in seedlings of many species of the orders Polypodiaceae, Polygonaceae, Chenopodiaceae, Caryophyllaceae, Umbelliferae, and Brassicaceae and concluded (296) that in these cases glutamine fulfilled the function more generally taken on by asparagine. More detailed study of the relative behavior of these two amides was made possible only subsequent to 1935 by the introduction (352) of a rapid indirect method of estimating glutamine in the presence of asparagine.

Mothes (234) reported that in response to ammonia feeding asparagine rather than glutamine accumulates in bean leaves. Viets *et al.* (360) found that in corn plants under ammonium nutrition both amides accumulate but asparagine is present in greater amount. Removal of the nitrogen supply resulted in amide depletion and under such conditions the asparagine disappeared more rapidly than the glutamine so that the relative amounts of the two amides was reversed. Street *et al.* (335) cultured detached potato sprouts in ammonium and nitrate culture solutions and subsequently in a nitrogen-free culture solution and studied changes in the concentration of both amides in the tissues. During nitrate culture, the initially low glutamine content of the roots remained fairly constant,



and the initially high content of the shoots gradually declined. The asparagine content of both shoots and roots underwent considerable fluctuation, sometimes exceeding the initial content, sometimes falling to a low level. During ammonium culture, both amides accumulated in root and shoot but the increase in asparagine was more marked so that it ultimately exceeded glutamine in the shoot system which had initially contained glutamine in greater amount. On transference of either ammonium or nitrate sprouts to a nitrogen-free culture solution, the content of both amides decreased, the asparagine being more rapidly depleted. Vickery *et al.* (358) have studied the nitrogen nutrition of *Narcissus poeticus* using plants grown in light and darkness, and supplied nitrate, ammonium, or nitrogen-free culture solutions. In all cases, the glutamine amide nitrogen content of the leaves remained small. Asparagine contents were enhanced by ammonium and particularly by nitrate feeding. The above results seem to indicate that in some plants asparagine represents a reserve of nitrogen arising during nitrate or ammonium assimilation and rapidly depleted under conditions of nitrogen-free culture. In these plants, glutamine is also present but seems relatively insensitive to the conditions of nitrogen nutrition. However, tomato plants, which also contain both amides in appreciable amounts, respond to ammonia feeding by accumulation of glutamine, no significant increase in the amount of asparagine occurring (76,351). Greenhill and Chibnall (146) and Curtis (89) have also reported, in response to application of ammonium sulfate, a pronounced accumulation and exudation of glutamine from the leaves of grasses.

Vickery and Pucher (350) have made a re-examination of amide accumulation in certain etiolated seedlings and their results indicate the variable behavior encountered. With seedlings of *Lupinus angustifolius*, marked asparagine accumulation occurs while the amount of glutamine present never rises above a trace. With *Vicia atropurpurea*, though both amides are present in similar amount in the soaked seed, asparagine accumulates during germination while the glutamine content does not increase significantly above the initial level. With *Cucurbita pepo*, though the amount of asparagine exceeds that of glutamine in the soaked seed and though both amides increase in amount during germination, glutamine accumulates more

rapidly and by the twelfth day significantly exceeds asparagine in amount.

Studies of the accumulation of amides during the starvation of detached leaves reveals a similar contrast in the amide metabolism of different species. Vickery *et al.* (356) recorded that asparagine is rapidly synthesized by excised tobacco leaves cultured in darkness. Under these conditions, the glutamine content only rises slightly, to an extent that might be expected from the liberation of the glutamine preformed in the protein hydrolyzed. On the other hand, when tobacco leaves are cultured in continuous light, both glutamine and asparagine are synthesized, the former to a somewhat greater extent than the latter. Wood *et al.* (405) found that leaves of Kikuyu grass resemble tobacco in showing, during culture in darkness, a marked accumulation of asparagine accompanied by only a small increase in the glutamine content. Rhubarb leaves, however, in either light or darkness synthesize only glutamine; asparagine remains absent or occurs in only very small amount (353). The leaves of barley (40) and oats (405) are intermediate in behavior, relatively large amounts of both amides accumulating during starvation. Yemm (410) studied the changes in concentration of both amides in barley leaves during 144 hours of culture in darkness. During the first 48 hours, glutamine accumulated more rapidly than asparagine. By 72 hours, however, the asparagine content exceeded that of glutamine and from then onward glutamine decreased in amount while asparagine continued to accumulate; by 96 hours asparagine was present in an amount almost double that of glutamine. The accumulation of ammonia became evident after 72 hours and during the final stage (96–144 hours) increased markedly as the content of both amides sharply declined. At 144 hours, glutamine was present only as a trace, but asparagine still exceeded its level recorded after 48 hours of culture. Mothes (238), reviewing the behavior of the two amides in etiolated seedlings and excised leaves, noted that in general glutamine attains its maximum sooner than asparagine and is apparently later converted into asparagine, so that in exhausted organs glutamine is absent though asparagine may be present in considerable quantity. Unpublished results obtained by the author and Miss G. M. Watson using potato leaves are shown in Figure 1. The course of amide accumulation and

depletion in the leaves cultured in darkness follows that observed by Mothes.

The conception of asparagine and glutamine plants adopted by Schultze is no longer tenable. In those cases in which asparagine constitutes almost the whole of the amide fraction, there is evidence for the occurrence of glutamine even though its concentration may normally remain at a very low level. Similarly, in a plant like

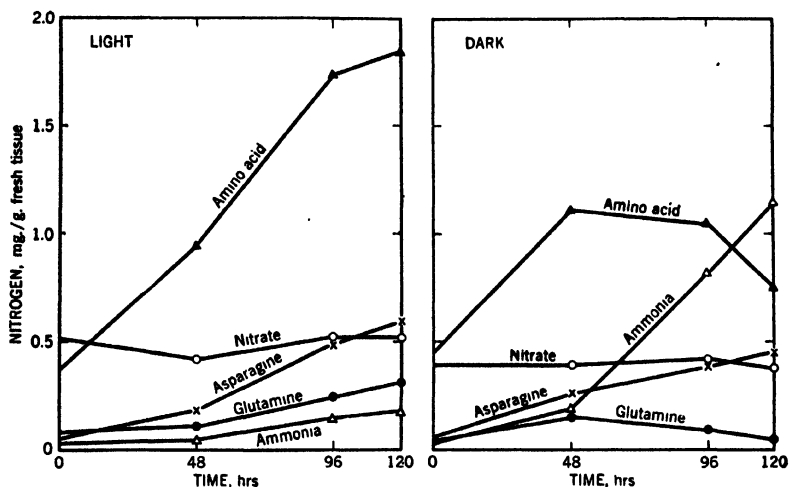


Fig. 1. Changes in concentration of fractions of nonprotein nitrogen of young potato leaves (King Edward) exposed to daylight (left) or kept in continuous darkness (right). Leaves detached from growing plants on August 14, 1944, and maintained with petioles immersed in distilled water for 120 hours.

beet, in which normally only glutamine can be detected, it has been shown (241) that under special conditions (anaerobiosis) the tissue is capable of synthesizing asparagine in place of glutamine. The possibility was early visualized (299) that there might occur in plants other amides in addition to asparagine and glutamine. Subsequently Vickery *et al.* (347,354) obtained data suggestive of this possibility. In one case, however—that of potato tuber tissue in which earlier data were suggestive of the occurrence of an amide similar to but not identical with glutamine (328)—subsequent work (331) has confirmed almost beyond doubt that asparagine

and glutamine constitute the whole of the amide fraction. This, combined with the complete lack of positive evidence for other amides, makes it almost certain that asparagine and glutamine are the only simple acid amides occurring in higher plants.

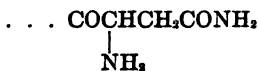
### *2. Availability of Respective Precursors*

Vickery *et al.* (351) concluded that, in general, in green plants one amide (in some cases asparagine and in other cases glutamine) functions for ammonia detoxication and that in such cases the function of the second amide is obscure. Later, however, they advanced the view (349) that if amide synthesis is regarded as an expression of the availability, respectively, of oxalacetic and  $\alpha$ -ketoglutaric acids, these being supplied in the course of the respiration of the tissues, wide variations in the behavior of different tissues or of the same tissue under different circumstances become intelligible. This hypothesis has not been subjected to detailed investigation since it is only recently that methods of estimating individual keto acids in plant tissues has received attention (94, 189, 196, 366–368, 373, 407). Through the Krebs citric acid cycle, oxalacetic acid and  $\alpha$ -ketoglutaric acid are interconvertible and therefore the postulated preferential accumulation of one of these acids implies that different reaction rates obtain for the successive steps in the cycle. This might conceivably lead to an explanation of the observed conversion of glutamine into asparagine as starvation proceeds if the former is regarded not only as a reservoir of nitrogen but also of  $\alpha$ -ketoglutaric acid, which it can yield to the cycle under conditions of carbohydrate depletion (75).

### *3. Amidases*

Shibata (308) as early as 1904 detected the presence in the mycelium of *Aspergillus niger* of an enzyme capable of splitting off ammonia from certain amides including asparagine; this has been confirmed by Schmalfuss and Mothes (292). A similar amide-splitting enzyme was shown to occur in yeast (100, 130, 136, 142, 198). Dernby (100), in his work with yeast, and Levene, Simms, and Pfaltz (204), studying intestinal erepsin, concluded that the amides were hydrolyzed by peptidases and that the acid amide group could be regarded as a peptide linkage in which one hydrogen atom of the

amino group was equivalent to the amino acid residue of a dipeptide:  $\text{—CO—NH—R}$ , where  $\text{R=H}$ . Grover and Chibnall (148), reporting the presence of an asparagine-hydrolyzing enzyme in the roots of germinating barley, similarly concluded that the enzyme concerned was a dipeptidase rather than a specific asparaginase. The enzyme isolated from yeast by Geddes and Hunter (136) was, however, found to split the amide group quantitatively from asparagine but to have but slight activity toward glutamine and to be quite inactive to all other acid amides tested; Grassmann and Mayr (142) were able to show that yeast asparaginase is an enzyme quite distinct from yeast dipeptidase and from aminopolypeptidase. The asparaginase of yeast was therefore exclusively an amidase incapable of acting as a peptidase. The enzyme was also shown to be highly specific depending for activity on the presence of the grouping:



In the presence of pyrophosphate, even the most active preparations were unable to attack glutamine. There was, however, evidence for the presence in fresh yeast autolyzate of a glutaminase in addition to asparaginase. The presence of glutaminases, quite distinct from asparaginase, was also demonstrated by Krebs (187) working on the mechanism of glutamine synthesis in various mammalian tissues. Schwab (305) demonstrated that barley seedlings yield a preparation active against both amides and postulated the existence in higher plants of two distinct amidases. Similar evidence (331) also indicates the presence of two distinct amide-splitting enzymes in potato tuber tissue. Chibnall (75) has demonstrated the synthesis of glutamine in the leaves of perennial rye grass in response to infiltration with ammonium  $\alpha$ -ketoglutarate or ammonium glutamate and thereby made it probable that a glutaminase is present. More recently, Archibald (10,11,12) has shown that the roots of tomato, zucchini, and perennial rye grass contain small but demonstrable amounts of an enzyme which hydrolyzes glutamine to ammonia *in vitro*, and that the enzymic activity of the extracts is greatly enhanced by previously feeding the plants ammonium glutamate.

Schwab (305) advanced the hypothesis that the quantitative

ratio of the two amides present in any given plant is determined primarily by the mass equilibrium ratio of the two enzymes and suggested that plants should be characterized as "asparaginase" or "glutaminase" plants according to the nature of their chief amide. There is as yet, however, no positive evidence in support of this conception. Furthermore, if a correlation between the amount of amide present and the amidase activity could be established, it would leave in dispute which was cause and which was effect.

It is tempting to consider how far the marked contrast in the stability of the two amides is of significance in determining their respective behavior. It is possible that, during the progress of starvation in detached leaves, the early decrease in glutamine concentration is due to the development of physical conditions adversely affecting the stability of its amide group. This naturally leads on to a consideration of the more difficult problem of the relative role of the two amides in protein synthesis as distinct from the factors accounting for their differential accumulation under conditions of protein breakdown.

Virtanen and Laine (372) have investigated how far the amide nitrogen of glutamine and asparagine can be utilized in transamination. Results, using crushed pea plants as the source of transaminase, indicated 31.1 and 10.7% transfer of the amino nitrogen of glutamic and aspartic acids, respectively, and 6.9 and 1.5% transfer of amide nitrogen from glutamine and asparagine, respectively. Doubt was cast, however, on the validity of the measurements with the amides since apparently some amide hydrolysis occurred so that the measured activity might have been that of the dicarboxylic amino acids liberated by hydrolysis. These results emphasize the greater activity of glutamic acid over aspartic acid in transamination, with also a possibility that glutamine itself may show appreciable activity. Steward and Preston (328,329) in a study of protein synthesis in discs of potato tissue concluded that the "unstable amides" (subsequently shown to be glutamine, 331) were reactive intermediates between reserves of amino acids and asparagine on the one hand and protein on the other. Glutamine concentration for any given rate of protein synthesis remained fairly constant and a rapid increase or decrease in the rate of synthesis was accompanied by depletion or accumulation of the amide. The data, previously discussed, from detached leaf experiments also

emphasize that the cessation of protein synthesis leads to an almost immediate accumulation of glutamine. The fact that both the amides are to be regarded as neutral reserves of available nitrogen in no way renders improbable the possibility that one of them may be more directly involved in the actual mechanism of synthesis. The behavior of glutamine in relation to protein synthesis must, however, be subjected to further investigation before it can be claimed that this amide plays any special function distinct from asparagine.

#### 4. *Glutamine as an Essential Growth Factor*

McIlwain *et al.* (220) reported that most strains of *Streptococcus hemolyticus* require glutamine for growth, and that the stimulating action of meat extract is due to its glutamine content. Growth of the streptococci took place, though less actively, if glutamate (at a concentration 100 times greater) was supplied instead of glutamine (219). This suggested that the activity of glutamate was due to its slow conversion to glutamine. More recently other pathogenic bacteria have been shown to require glutamine or to be markedly stimulated by its presence and in no case could the glutamine be replaced by asparagine. Pollack and Lindner (268, 269) have also reported a marked stimulation of growth and lactic acid production in *Streptococcus lactis* and *Lactobacillus arabinosus* when supplied glutamine, and advanced the view that glutamine is essential for the synthesis of cellular proteins. A low activity of glutamic acid was also recorded by Lyman *et al.* (215) and explained as due to its action as a precursor of glutamine, a contention supported by the observation that ammonia stimulated lactic acid production in presence of glutamic acid but was without effect in presence of glutamine. The growth stimulation which can be achieved with L-glutamic acid, D-glutamic acid,  $\alpha$ -ketoglutaric acid, and glutathione is also apparently dependent upon the conversion of these substances into glutamine (150). Although the mechanism of action of glutamine in these microorganisms is as yet obscure, the fact that it is for them an essential growth substance emphasizes the possibility that it may, in higher plants, also play some special role in metabolism.

The above discussion reveals certain important similarities and

differences in the behavior of asparagine and glutamine in the cells of higher plants. The evidence suggesting that glutamine is particularly involved in protein synthesis is inconclusive and it remains for future work to show how far the unique position of glutamic acid in amino nitrogen metabolism is sufficient to explain the apparently special metabolism of its amide.

## V. Protein Regulation and Path of Synthesis

### A. AMINO ACID HYPOTHESIS

#### 1. *Factors Regulating Protein Synthesis in the Plant*

The interpretation of data relating to the factors involved in protein regulation must take account of the possibility that both enzymic and nonenzymic ("template") syntheses of protein may be proceeding simultaneously in the same cell and that, therefore, simple correlations are not to be expected between either protein content or the rate of protein synthesis and the concentration of such possible simple precursors as amino acids. Two main conceptions as to the path of protein synthesis have, however, influenced the views put forward as to the nature of protein regulation. The first conception, based on the peptide hypothesis of Hofmeister (160) and Fischer (122), visualizes the separate synthesis of the amino acids and then their step-by-step condensation through simple peptides to polypeptides to proteins. The second conception that proteins might be synthesized by some alternative mechanism was first seriously advanced by Abderhalden (1), but has never been set out in explicit terms so that it does not rank in clarity with either the proteinase or template hypotheses. This alternative view involves considering the amino acids as secondary products formed only by protein hydrolysis and postulates that the protein molecule arises by *en bloc* condensation of units different from and probably simpler than the amino acids.

Evidence in favor of the view point that protein synthesis in plants takes place by amino acid condensation has been summarized by Petrie (260,261) and by Wood (401). Petrie (260), discussing the possibility of an alternative path of synthesis, admitted that it was impossible to ascertain with certainty the mechanism involved but in a later review (261) he pointed out that the amino acid hypothesis was more clearly defined and had the virtue that it enabled



possible synthetic mechanisms to be discussed and suggestions to be made as to the mode of action of determining factors, whereas all alternative hypotheses were of a very speculative nature and were vague with regard to the stages traversed. Certainly the evidence that primary synthesis of amino acids takes place in plants would receive a teleological explanation if protein formation takes place by amino acid condensation, and the linkage of nitrogen and carbohydrate metabolism would be located at the synthesis of amino acids. Increases in amino nitrogen, above those to be expected from the extent of protein hydrolysis, have been observed by various workers (75) but a complete quantitative analysis of the individual amino acids synthesized has not been undertaken in any instance and only in the cases of glutamic acid, aspartic acid, and possibly arginine is there positive evidence for their primary synthesis. It, therefore, remains possible that the identity of the accumulated amino acids is determined by the availability of suitable nitrogen-free precursors and that the whole of the amino fraction represents a labile store of nitrogen, the utilization of which for protein synthesis does not involve direct condensation of its constituent amino acids. The concomitant disappearance of amino acids and synthesis of polypeptides and proteins such as occur during the ripening of seeds and the development of storage organs have been regarded as evidence in favor of synthesis by amino acid condensation. These changes are, however, equally well explained by regarding the amino acids as nitrogen reserves, their disappearance occurring by oxidative deamination and utilization of the liberated ammonia in synthesis. Furthermore there is considerable evidence that certain amino acids are the precursors of such biochemically important substances as glutathione, choline, nicotinic acid, thiazole, and purines; therefore their primary synthesis could, in the absence of evidence to the contrary, be regarded as related to the formation of these derivatives. The observation that certain amino acids are indispensable for higher animals still poses the question of to what purpose these are utilized in the organism. The demonstrations in such cases that tryptophan can be replaced by indolelactic and indolepyruvic acids and that phenylalanine can be replaced by phenylpyruvic acid only serve to emphasize that the demand is for a particular carbon chain rather than for the amino acid itself. Much further work is required to examine how far the nature and

amounts of the amino acids synthesized by plants are correlated with the establishment of that proportionality within the amino acid fraction demanded by the amino acid condensation hypothesis.

Chibnall (75) has, however, adopted the general viewpoint that amino acids are the immediate building stones used in protein synthesis and that the concentration of amino acids will be a factor controlling the rate of protein synthesis. On the evidence that  $\alpha$ -keto acids rather than hexoses are the immediate precursors of amino acids, Chibnall has suggested that the Paech hypothesis (254) be rephrased to read "in the intact plant cell, the control toward protein synthesis or decomposition will take place through the amounts of a necessary series of  $\alpha$ -ketonic acids and of active nitrogen, in that an increase or decrease of the component present in minimum amount will bring about equivalent protein changes." Direct experimental support for such a hypothesis is lacking and furthermore the verified existence in higher plants of only three  $\alpha$ -keto acids (pyruvic, oxalacetic, and  $\alpha$ -ketoglutaric) requires a cautious approach even to the view that amino acids are synthesized from a corresponding series of  $\alpha$ -keto acids, particularly since there is evidence, that in animal tissues glutamic acid can arise from histidine (2) or proline (37,391) and that in *Neurospora* indole and L-serine are the precursors of tryptophan (341).

Petrie (261) has applied the concept of a "steady state" relationship (67) to the interpretation of the data from nitrogen metabolism studies in plants, the nitrogen fractionation in the cell being regarded as having attained a "steady state" when the concentrations of reactants and end products and the rate of energy consumption are all constant. Petrie, while conceding that such a steady state is in reality never attained in the nitrogen metabolism of the growing plant, considers that the data obtained with plants grown under carefully controlled conditions can be interpreted on the assumption that the nitrogen metabolism reaches a "drifting steady state" under which change in the determinants of the state is so slow that a succession of states results, each of which is inappreciably removed from a steady one. The protein level of the cell is thus considered to be determined by the concentration of reactants and also by certain parameters relating directly or indirectly to the energy release. If the concentration of reactants is one of the determinants of protein level, these reactants should be

identifiable by the existence of significant correlations between their concentration and the protein level. This approach underlies the examination by Wood, Petrie, and co-workers of the evidence for a mass action relationship between amino acid concentration and protein level. Petrie and Wood (263,264) found that, when conditions approached a steady state relationship, the protein nitrogen level was a function of the total free amino nitrogen and the water content when these three variables were expressed on a dry weight basis. The results could be expressed by the equation:

$$P = a + b_1A - b_2A^2 + b_3U$$

where  $P$  = total protein nitrogen,  $A$  = total free amino acid nitrogen,  $U$  = water content, and  $a$  and  $b$  are constants. Such a system is to be regarded as one maintained at a nonequilibrium condition by a continuous expenditure of energy and one in which reduction of the value of  $U$  results in protein hydrolysis. Thus there appeared to be a definite relationship between protein and total free amino acid contents although this did not conform to a simple mass action relationship of the kind:

$$[P] = K[A_1][A_2][A_3] \dots [A_n]$$

A similar interrelationship was also observed by Wood (400) and the existence of a definite steady state relationship between protein nitrogen, amino acid nitrogen and water content formed the basis for concluding that proteins are formed, in higher plants, by direct condensation of the amino acids within the cell rather than along an alternative path.

The association of increased water content with increased protein content recorded by Wood and co-workers is in agreement with some earlier observations (84,95,133,235,236,246). It should be emphasized, however, that such a relationship does not always obtain. The accumulation of high protein content in seeds is associated with the onset of desiccation (109). Study of the effect of relative humidity and temperature on the composition of tomato and apple plants (247) has shown that the ratio of protein nitrogen to total nitrogen increases with increasing desiccation. Wadleigh and Ayers (380), noting a high value for protein nitrogen in bean plants grown under conditions of severe physiological drought.

have interpreted the reduced growth rates as due to inhibition of swelling of the protoplasmic proteins and decreased vacuolation rather than to any impairment of protein synthesis associated with reduced water content. Furthermore Wood, Petrie, and co-workers recognize that the simultaneous hydrolysis of protein and accumulation of amino acids in detached leaves under starvation conditions cannot be explained on water content changes in the sense set out by Petrie and Wood (263,264) nor, at least during the early stages of starvation, on lack of respiratory energy for maintaining the nonequilibrium state. They (263) noted, however, that the increases in total free amino acid content which occurred with decrease in water content did not involve any increase in cystine content and later (402), using leaves of rye grasses starved in air, observed a parallel decrease in protein sulfur and protein nitrogen, the former accompanied by an equivalent increase in inorganic sulfate sulfur. The fact that during protein catabolism the level of soluble organic sulfur (cystine, methionine, glutathione) remained constant suggested that the sulfur-containing amino acids arising from protein degradation had suffered oxidation. Using leaves of Sudan and Kikuya grasses Wood *et al.* (404) obtained confirmatory evidence that the decrease in protein nitrogen and accumulation of amino acid nitrogen which occurred when the leaves were starved in air was associated with oxidation of cystine at a greater rate than it was being formed. The resulting disproportionality among the free amino acids was regarded as the cause of the continued protein hydrolysis. The protein-sparing action of high carbohydrate supply could on this basis be interpreted as due to the action of carbohydrate as an adequate source of nonnitrogenous precursor able to condense with ammonia and sulfhydryl compounds for cystine synthesis. In a study (403) of the change in concentration of certain selected amino acids during starvation of grass leaves in air, it was found that the content of each amino acid reached a maximum and then declined. Comparison of the maximum amount of each acid accumulated with the yield to be expected from the amount of protein hydrolyzed—using Chibnall's data for the amino acid composition of grass leaf proteins—yielded data supporting the view that there exists a steady state relationship between proteins and amino acids but that this may be disturbed by changes in the composition of the amino acid fraction resulting

from preferential oxidation of certain amino acids (particularly of cystine and to a less extent of glutamine and arginine). Abnormal proteolysis alongside a high content of available carbohydrates has long been regarded as a symptom of sulfur deficiency in tomato (242,249). Eaton (107,108) noted a similar condition in sulfur-deficient sunflowers and soybeans although in these cases, despite abundance of reserve carbohydrates, there did occur a lower concentration of reducing sugars and sucrose than in the controls. Eaton considered that very little of the soluble organic sulfur of the sulfur-deficient plants was in the form of cystine or glutathione and that inability to synthesize sulfur-containing amino acids prevented normal synthesis of protein via amino acid condensation. It has, however, been pointed out (261) that the high values for soluble organic nitrogen in the stems of sulfur-deficient plants, as recorded by Eaton, are associated with a high total nitrogen content and do not necessarily indicate the occurrence of abnormal proteolysis. Lugg and Weller (214) have, however, obtained evidence indicating that, during seed germination, the amount of available methionine may be the limiting factor in protein synthesis.

The work of Viets *et al.* (360) shows that in the corn plant despite the increasing water content and increasing amino nitrogen content resulting from ammonium nutrition there was, in the presence of abundant carbohydrate, little change in protein content. The general conclusion was advanced that factors more decisive than water content, amino acid level, or potential ammonia compounds and abundant carbohydrate are concerned in the regulation of the protein level. It was, however, observed that the marked increase in amino acid nitrogen was not paralleled by increase in the basic amino acids, so that the proportion of basic to total amino acid nitrogen was low compared to the basic amino acid content of corn proteins. It is therefore possible to postulate that the high amino acid to protein ratio was due to disproportionality of certain essential amino acids. A number of workers (309,310,313,335,355) have noted high values for amide and amino nitrogen in the roots of ammonia-grown plants and, by contrast, in the roots of nitrate-grown plants, a small content of these fractions and a correspondingly high content of "residual nitrogen" (presumably largely proteose and polypeptide nitrogen). This very marked contrast between the protein to amino acid ratio in the two series has led

to the suggestion (313) that the route of protein synthesis may be somewhat different according to the nitrogen utilized. This suggestion is worthy of re-examination in the light of the work of Viets *et al.* cited above.

Phillis and Mason (265), in studies of the nitrogen partition in the cotton leaf, concluded that the *partition index* (insoluble nitrogen as a percentage of the total nitrogen) was largely determined by the degree of saturation of the tissues with nitrogen and suggested that the concentration of crystalloid nitrogen played a part in regulating the content of insoluble nitrogen. Further work (226, 227, 266) emphasized, however, that the ratio of crystalloid nitrogen to protein nitrogen was subject to considerable variation depending upon the level of nitrogen nutrition. The concept of a relationship between crystalloid and protein nitrogen contents was, however, retained, the drift in the ratio with increasing nitrogen supply being considered to follow broadly the drift of apolar adsorption (70) with increasing concentration. On this basis it was suggested that adsorbed crystalloid nitrogen was the main factor in determining the protein level. The data of Richards (276) were regarded as supporting this hypothesis. The reduced protein levels found to be associated with phosphorus and potassium starvation (227) were regarded as due to reduction of adsorptive surface by these nutritional deficiencies. Richards (278) has, however, subjected these data of Mason and Phillis to criticism and challenged the evidence for statistically significant correlations between crystalloid and protein nitrogen.

The attempts, therefore, to establish a correlation between amino acid and protein contents have been inconclusive. The changing ratio of amino acid nitrogen to protein nitrogen has been explained either by postulating changes in the composition of the amino acid fraction (261, 401) or on the basis that the concentration of amino acids at the seat of protein synthesis is not indicated by the total amino acid content of the cell (266, 383). It is hardly to be expected that a heterogeneous fraction such as total amino acid nitrogen should be simply related, over a wide range of values, to the heterogeneous total protein fraction, even if protein synthesis does proceed by amino acid condensation. Considering the complexity of protein molecules, it is hard to conceive of a mass action relationship of the type suggested by Petrie and Wood (263, 264) even if we assume

at the seat of synthesis a proportionality in the amino acid fraction corresponding to that required by the protein molecule being synthesized. Furthermore it is possible that, under certain conditions, the total amino acid nitrogen would be positively correlated with the rate of protein synthesis if the amino acid fraction was acting as the principal source of readily available nitrogen quite irrespective of the mechanism of synthesis.

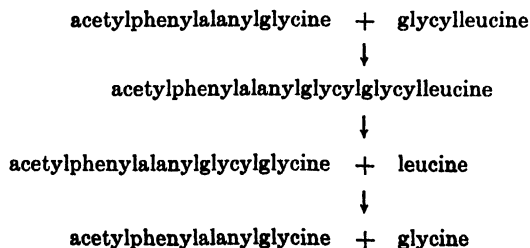
## 2. Attempts at *in Vitro* Synthesis of Protein

The chemical synthesis of peptides from amino acids and the fact that the greater part of the nitrogen of proteins is recovered as amino acid nitrogen when proteins are hydrolyzed by acids, alkalis, or proteolytic enzymes have invited attempts to achieve *in vitro* the enzymic synthesis of polypeptides and proteins from simple mixtures of amino acids. The enzymic synthesis of proteinlike material (plastein) from protein digests was first reported by Wastaneys and Borsook (388,389). Subsequently a number of workers (41,81,139,151,228,229,378) have reported that, on oxygenation and in the presence of a proteinase, certain protein digests exhibit a reversal of proteolysis. The plasteins of Wastaneys and Borsook, however, do not have the molecular dimensions of proteins (123-126). Furthermore the reported syntheses of protein have not proved capable of repetition in the hands of other workers and attention has been drawn to the fact that decreases in the nitrogen soluble in trichloroacetic acid are not accompanied in these digests by corresponding decreases in Van Slyke amino or basic nitrogen (208,209,333,334). The evidence for an *in vitro* synthesis of protein from protein digests under the action of proteinases is therefore highly controversial in nature.

The aeration of or addition of oxidizing agents to the protein digests by Wastaneys, Borsook and others in their work on plastein formation was based on the view that the reduced form of the enzyme was active in hydrolysis and the oxidized form in synthesis. This seemed to agree with the earlier reports that in plant tissues, anaerobic conditions modify protein metabolism in the direction of proteolysis rather than synthesis (38,68,234,237). Schulze (302) claimed to have detected in plants a regulator of protein synthesis chemically related to cysteine which activated proteolysis in the reduced form ( $-SH$ ) and inhibited it in the oxidized form

(—S—S—). Mothes (237) also considered that oxygen affected the balance between synthesis and hydrolysis through its effect on the sulfhydryl activators of plant proteinases. Certainly recent work has shown that the activity of papain as judged by rate of hydrolysis of protein or synthetic substrates is dependent upon free sulfhydryl groups and aeration or treatment with oxidizing agents inactivates the enzyme; but the conception underlying the hypotheses discussed above must be regarded as highly speculative since normally activation affects the rate of reaction and not the position of equilibrium. Furthermore, Paech (254) has questioned whether the proteolysis observed to occur under anaerobic conditions was not related to injury or death of the cells rather than to activation-inhibition changes in the proteinases; and Wood *et al.* (403–405) have reported, in contrast to earlier workers, only a very slow rate of protein breakdown in detached grass leaves starved in nitrogen, and have made the speculative suggestion that there exists a static pattern operative under anaerobic conditions which protects the proteins from enzymic hydrolysis.

The work of Bergmann *et al.* (29–32) has demonstrated that simple peptides can be synthesized from amino acids by cysteine-activated proteinases. These *in vitro* syntheses were effected under conditions which rendered the peptides insoluble and thereby removed them as formed from the sphere of reaction. It was therefore postulated that in living organisms the synthetic reactions catalyzed by proteinases are made possible by a coupling of the proteolytic systems with other equilibrium systems. Behrens and Bergmann (27) have shown that cysteine-activated papain will not attack glycyl-L-leucine alone, but will do so in presence of acetylphenylalanylglycine. This action was explained by postulating the following series of reactions involving the initial synthesis of a tetrapeptide, followed by its degradation to a tri- and then a dipeptide:





A similar explanation will probably cover the many recorded cases in which peptides resistant to proteolytic enzymes are hydrolyzed by the same enzymes in presence of proteins. These experiments not only demonstrate peptide synthesis but show how the specificity of the proteolytic enzymes could determine the periodicity of amino acids along the polypeptide chains as demanded by the Bergmann-Niemann hypothesis (34,35). The flexibility of this specificity as it is affected by different activators allows also the synthesis of variants upon the general protein pattern demanded by the heterogenous nature of the protein fraction of living cells (33).

The view that protein synthesis takes place by amino acid condensation—that it is a reversal of the process of proteolysis—is supported by the facts that the protein molecule is built up of amino acid residues united by peptide linkages, that amino acids can act as sources of nitrogen for protein synthesis, and by the evidence that each step in the hydrolytic cleavage of proteins by enzymes is a reversible reaction, although the position of equilibrium lies almost entirely on the side of degradation. Claims for *in vitro* enzymic syntheses of proteins cannot, however, be regarded as satisfactorily established. The data obtained from studies of nitrogen metabolism in living plant material, and which have been used in support of the amino acid hypothesis, are seen in all cases to be capable of alternative interpretation.

#### B. ALTERNATIVE PATH HYPOTHESIS

The second conception of the nature of the precursors involved in protein synthesis has developed from the Abderhalden diketopiperazine hypothesis first enunciated in 1924 (1). This hypothesis in its original form was, however, severely criticized by Grassmann (141) and is now no longer tenable. Block (44–46) noted the constancy of the molecular ratios of the basic amino acids in keratins and in mammalian and avian serum proteins, stressed the primary importance of the basic amino acids in the genetic and embryological development of these tissue proteins, and put forward the hypothesis that they are built upon or around an “Anlage” of relatively fixed proportions of arginine, histidine, and lysine. This “Anlage” was visualized as constituting a central nucleus around which the additional amino acid residues became grouped. This con-

ception, which differs from the normal polypeptide hypothesis and raises the question of the origin of the central "Anlage" by an alternative path to simple amino acid condensation, has however, been severely criticized by Bergmann and Niemann (35). Alcock (4) has examined existing amino acid analyses of proteins and plotted the percentage of each amino acid in a protein against the frequency of its occurrence at that percentage in different proteins and found pronounced norms. These peak percentages, he considered, represented the percentage content of the different amino acids in a primitive protein. This primitive type of protein was postulated to be common to all living cells, but to be capable of undergoing further differentiation into the more complex particular proteins characteristic of individual species and tissues. Alcock considered that the synthesis of the primitive protein could be visualized as commencing with the formation of a simple unit possibly by condensation of formaldehyde with ammonia or nitrous acid, and that this unit then polymerized and underwent internal differentiation to give a molecule containing amino acid residues in definite proportions. Jannsen (170) has visualized protein synthesis as taking place by the linking up of half glucose molecules followed by the insertion of nitrogen atoms from previously existing nucleoproteins.

Paech (254), as a result of an extensive review of the literature and from his own researches, was led to adopt the view that it is the concentrations of active monoses (hexoses) and of ammonia in the cell which regulate the rates of protein formation and breakdown, the relationship obeying the mass action law within the limit set at one extreme by the maximum storage power of the cell for protein and at the other by the minimum amount of protein necessary for the maintenance of protoplasmic structure. Chibnall (75) has criticized the experimental basis on which the Paech hypothesis is founded and has also pointed out that it requires, contrary to general experience, that large amounts of monoses and "active" forms of nitrogen (ammonium and amide nitrogen in particular) should never be present together in the same cell and that, as soon as a suitably high level of both components is available, regardless of other conditions, protein should be 'synthesized. Numerous workers (6,40,147,276,356,357,404,406,409) have shown that the protein level is by no means necessarily related to sugar concentration and that under certain conditions proteolysis rather than synthesis occurs when the "active nitrogen" content is increasing and

the sugar concentration is at a high level. Amos and Wood (6) suggest that carbohydrate is of significance in protein metabolism as a respiratory substrate and as the source of nitrogen-free precursors for amino acid synthesis and that the nitrogen metabolism will be unaffected by wide variations in carbohydrate content if the formation of glycolytic products from hexose follows the Michaelis and Menton equation:

$$R = KE \left( \frac{S}{S + K_m} \right)$$

where  $R$  is rate of formation of glycolytic products and  $S$  is hexose concentration. At high active hexose concentrations the rate of glycolysis ( $R$ ) will then remain practically constant and independent of the value of  $S$ . The work of Wood and Petrie (406), designed to test the Paech hypothesis, showed that the carbohydrate content did not affect the protein to amino acid relationship. The sucrose content of all sets of leaves examined was extremely high and it was therefore postulated that the rate of glycolysis would be unaffected by changes in carbohydrate content if the furanose to  $\alpha$ -keto acid relationship was of the Michaelis type. The hydrolysis of protein under starvation conditions would be explained as due to a lower rate of  $\alpha$ -keto acid formation and hence of amino acid synthesis associated with the lowered rate of glycolysis.

Gregory and Sen (147) observed that the relationship between proteins and amino acids did not obey the mass action law and therefore concluded that the two processes of protein synthesis and degradation were probably distinct and were catalyzed by separate enzyme systems. Protein synthesis was regarded as a process distinct from proteolysis and not as a reversal of the latter. The amino acids resulted from protein degradation; they suffered deamination and the ammonia combined with carbohydrate derivatives to give amides. Protein was synthesized directly or indirectly from the amide nitrogen. Steward *et al.* (327-330) pointed to the possibility that protein arises directly from sugar (or sugar derivatives) and ammonia rather than that the individual amino acids are separately synthesized and then condense via peptides to proteins. The behavior of the easily hydrolyzable amides of potato tissue was interpreted as indicating that they might represent unstable intermediates formed from sugar and ammonia and give rise by further

condensation directly to proteins. The following sequence was therefore put forward to represent the path of synthesis:

amino-acid  $\longrightarrow$  ammonia  $\longrightarrow$  "unstable amides"  $\longrightarrow$  protein

The identification of the unstable amide fraction of resting potato tubers with glutamine (331) makes it improbable that protein arises directly from this fraction. The apparent key position of glutamine in protein synthesis, however, suggests either that it plays a special part in amino acid synthesis or that it is involved in some, as yet not clearly defined, alternative path. Albaum and Cohen (3) have questioned that the significance of transamination is limited to interconversion of amino acid substrates in such a way as to ensure the proper proportionality of amino acids demanded by the amino acid hypothesis. As already indicated, they have drawn attention to the work of Linderstrøm-Lang (207) and of Herbst and Shemin (155) on the synthesis of peptides from glyoxals and amino acids or from keto acids and amino acids by a mechanism involving transamination. Their own data show that, in germinating oat seedlings, the transaminase activity increases ahead of the increase in protein synthesis and that there is a general relationship between protein synthesis, carbohydrate metabolism (presumably supplying  $\alpha$ -keto acids or possibly glyoxals), and transamination. Doermann (105) in a study of the arginine inhibition of a lysine-less strain of *Neurospora* has also cited the suggestion of Linderstrøm-Lang (207) and Kalckar (173) that aldehydes may be involved in peptide synthesis and has postulated that in the normal strain synthesis of lysyl peptides may not involve lysine itself (Scheme II, p. 406).

### C. RESPIRATION AND PROTEIN SYNTHESIS

Hoffman (159) noted that the respiration of potatoes and of germinating barley could be correlated with their nitrogen content. Relationships between protein synthesis and respiration of carbohydrates and between respiration rate and protein content have also been noted with barley leaves (147,276). Petrie and Williams (262) and Wood (399) were able to correlate respiration and protein content in grass leaves taken at various stages of the life cycle and Wood (400) emphasized that this correlation was most marked between respiration rate and cytoplasmic protein, suggesting that chloroplast protein, which behaves in many respects like storage protein, was less intimately related to respiration. During the pre-

storage stage, the drift in the respiratory activity of pears is very closely related to their protein content (174). During storage, rise in protein nitrogen is also associated with enhanced respiration but the climacteric rise although accompanied by an increase in protein nitrogen is out of all proportion to it. A similar lack of correlation between respiration rate and protein content has been repeatedly observed during the climacteric rise in the respiration of starving leaves.

Steward *et al.* (327,329,330) have shown that, in actively metabolizing discs of potato tissue, there is an intimate relation between protein synthesis and a component of the system involved in carbon dioxide production. Similarly a number of workers (348,404,405,410) have studied the source of the carbon dioxide given off by detached leaves and have shown that protein is early drawn into the respiration. The amino acids are oxidatively deaminated, amides accumulate, and the nitrogen-free residues of the amino acids are completely oxidized with liberation of carbon dioxide. Gregory and Sen (147) considered that the amino acids arising from protein degradation in barley leaves similarly suffered oxidative deamination. Steward and co-workers noted that the salt and oxygen treatments which stimulated respiration and protein synthesis also led to enhanced activity of the aerobic oxidase and therefore postulated that the oxidative deamination of the amino acids constituted, through the activated oxidase, the process linking protein synthesis, respiration, and the effect of salts and oxygen tension. A marked correlation between amino acid content and respiration rate is claimed by some workers (137,303,317) but Russell (286) has concluded that the earlier reports of the stimulation of respiration by amino acids should be treated with caution—that they were probably due to coincident bacterial contamination. More recently, however (400, 404), a high positive correlation between respiration rate and amino acid content seems to have been confirmed.

The work, outlined above, on the relationship of protein metabolism to respiration has been variously interpreted. The high respiration rate associated with a high protein level in the cell has been regarded as due to correlation of respiration rate with the high amino acid content associated with a high level of protein. Such an explanation requires the existence of a correlation between amino

acid and protein contents, and implies that respiration proceeds entirely via amino acid oxidation and that the rate of deamination is simply related to the amino acid concentration. Steward and co-workers have shown that protein synthesis is correlated with a component of the respiration which probably proceeded via amino acid deamination but that the magnitude of this appears to be determined primarily by the activity of the deaminating enzyme rather than by the amino acid content. Gregory and Sen have postulated that the rate of carbon dioxide production is controlled by operation of a protein cycle. The rate of protein synthesis is correlated with the rate of deamination and the supply of amino acids for deamination is regulated by the rate of protein hydrolysis or the supply from outside the cell. The speed with which this cycle is operated controls ultimately the rate of carbon dioxide production. This general concept of a protein cycle is strongly supported by the work of Schoenheimer and co-workers (293,294). When animal cells were supplied with single amino acids the amino groups of which were labeled with  $^{15}\text{N}$ , in a remarkably short time almost all the amino acids recovered by hydrolysis of the body proteins contained  $^{15}\text{N}$ . These data suggest that all the body proteins are continually breaking down and being resynthesized and that the amino nitrogen of the supplied amino acid is available for synthesis of all the units of the protein molecule.

The correlation of protein synthesis with the rate of deamination suggests that the simultaneous liberation of ammonia and energy does, in the presence of a supply of glycolytic products, regulate the synthesis. Wood (399,401) and Petrie (261) have however suggested that, in their own and others workers' researches, the correlation between respiration rate and protein nitrogen is due to the fact that the latter is a measure of protoplasm and hence of the respiratory centers in the cell. This view requires that protein content should control respiration rate and that any enhanced respiration associated with an enhanced rate of protein synthesis should be correlated with change in total protein content rather than with the rate of synthesis itself. The results of Steward *et al.* showed however that, in potato tuber tissue, the respiration rate could be correlated with the rate of synthesis rather than with the change in total protein content. Thus it was found that:

$$Y = 197.6 + 41.56x$$

where  $Y$  is total respiration and  $x$  is soluble nitrogen converted to protein. These results indicate that, while some data relating protein and respiration may be explicable on the basis of protein content as an index of the respiratory capacity of the cell, yet in actively metabolizing cells changes in respiration rate can be directly related to changes in the *rate* of protein synthesis.

There is considerable evidence in favor of the reversibility of many enzymic reactions although *in vitro* they proceed almost to completion in the direction of release of free energy. The conditions obtaining in the living cell, however, rarely correspond to the equilibrium state. Such hypotheses as that introduced by Kursanov (199) and Oparin (253), which postulates that the balance between synthesis and hydrolysis is determined by the ratio of the amount of enzyme adsorbed on protein surfaces to the amount of free enzyme, seem extremely speculative. Similarly the conception of a hormonal control of protein synthesis advanced by Chibnall (75) has now been criticized by Wood (401) on the grounds that recent studies (266,383) have demonstrated that mature, *detached* leaves are able under suitable conditions to effect protein synthesis. The reductive amination of  $\alpha$ -keto acid to amino acids and the formation of peptides from amino acids are both reactions by which the free energy of the system is increased. If the final stage in protein synthesis is the reverse of denaturation it will also be an energy-consuming reaction. These reactions therefore must be driven by energy derived from linked reactions and respiration must be related to protein synthesis insofar as the latter involves energy-consuming reactions.

The question of energetic coupling in biological synthesis has been reviewed in detail by Kalckar (173) and Lipmann (210). Phospho, acetyl, and amino groups are of special interest biologically in that they are "weak" linkages—showing a high tendency to burst or escape away. These properties are associated with a high group potential; the groups are held by energy-rich bonds. The phospho group is of particular interest in that its group potential varies according to the nature of the linkage through which it is bound. Furthermore, certain energy-liberating reactions have been shown to be associated with the generation of energy-rich phosphate bonds, and energy-consuming reactions with destruction of such bonds.

The significance of phosphorylation in carbohydrate metabolism can now be related to the existence of phosphate bonds of different energy content. The energy-rich phosphate bonds ( $P-O\sim P$ ,  $N\sim P$ ,  $O=C-O\sim P$ , and  $C=C-O\sim P$ ) are generated from energy-poor phosphate bonds (ester or inorganic linkages) in reactions involving a decrease in free energy and are in turn capable of giving up their energy to drive synthetic reactions involving increase in free energy. The natural occurrence of arginine phosphate in invertebrates (230) and the demonstration that its phosphate bond energy can be translated into muscular work suggest that the formation of amino acid phosphates with the energy-rich  $N\sim P$  may be of significance in peptide synthesis. Lipmann (210) has also pointed out that the acyl phosphates of the amino acids, which contain the grouping  $O=C-O\sim P$ , may be similarly involved. Borsook and Huffmann (50) have calculated that the synthesis of leucylglycine requires 7520 cal., and Lipmann (210) estimates that the average energy present in an energy-rich phosphate linkage amounts to 9000–11,000 cal. The demonstration that phosphorylation is involved in protein synthesis awaits the isolation and identification of phosphorylated amino acids or other protein intermediates and correlation of protein content changes with changes in energy-rich phosphate. It is already well established (227,276,395) that lack of phosphorus in plants is associated with a reduced protein level in the cell, and that with increasing phosphorus supply there is an increase in the rate of protein synthesis and in the protein to amino acid ratio.

The importance of potassium in the synthesis of protein is indicated in a number of papers (5,73,217,311,326). Increasing potassium, in general, leads to an enhanced rate of protein synthesis and a more complete utilization of the available nitrogen supply. Gregory and Sen (147) and Richards (276) have shown that potassium deficiency is associated with a reduced protein synthesis and an accumulation of amino acids; Steward and Preston (329), finding potassium absorption to be associated with enhanced respiration and protein synthesis, have suggested that potassium activates the aerobic oxidase system.

Gilyarovskii and Chernov (138) and Parks *et al.* (256) have noted that boron facilitates nitrogen absorption and its conversion into organic form. Schropp and Arenz (295) have found boron



deficiency to be associated with inability to synthesize protein and the development of symptoms resembling ammonia toxicity. Later, Briggs (56) showed that boron deficiency was in fact associated with ammonia accumulation and a high ratio of soluble organic nitrogen to protein nitrogen suggesting impairment of protein synthesis. Boron toxicity was associated with an abnormally low ratio of soluble organic nitrogen to protein nitrogen, storage protein accumulating in the cells. Reinders (275), using potato discs in aerated water, noted that auxin caused water uptake and apparently enhanced utilization of carbohydrate in respiration. Commoner *et al.* (82,82a) obtained evidence that indoleacetic acid promotes cell elongation not by affecting cell wall extensibility as suggested by Went and Thimann but by regulating water and salt absorption. Northen (251) suggested that auxins caused dissociation of cellular proteins into smaller units and that this led to increased swelling and polysaccharide hydrolysis. That the enhanced water uptake resulting from auxin application to potato discs is not associated with any increase in osmotic pressure by carbohydrate hydrolysis is indicated by the work of van Overbeek (346). Van Overbeek noted an increase in the nonosmotic factor in water uptake ("active pressure"), which is related to respiration and compared the effect of auxin to potassium-induced water absorption (330). The latter is related to respiration and protein synthesis and seems to involve activation of the system deaminating amino acids. Van Overbeek therefore tentatively suggested that under these conditions the enhanced rate of deamination may have caused accumulation of indoleacetic acid from tryptophan, and that this might have been the cause of the enhanced water uptake. Frey-Wyssling and Blank (129) have shown that cell elongation is accompanied by a marked increase in the protein content of the cell, and Avery and Linderstrøm-Lang (17) have obtained results suggesting that auxin may be concerned in the activation of proteolytic enzymes. These suggestions that auxin is concerned in the regulation of protein metabolism and the fact that indoleacetic acid will at least partially replace boron in the young cotton plant (106) suggest that boron is concerned in auxin formation and that the boron deficiency symptoms, particularly those relating to disturbance of nitrogen metabolism, may, at least in part, be due to lack of the hormone.

The researches hitherto designed to reveal the path of protein

synthesis have failed to yield critical data. Introduction of analyses of certain individual amino acids into a general study of nitrogen metabolism (403)—if they could be extended to cover the main part of the amino acid fraction and if they could be accompanied by parallel determination of organic acids, particularly  $\alpha$ -keto acids—should yield valuable data. Furthermore the use of the stable isotopes  $^{13}\text{C}$  and  $^{15}\text{N}$  introduced, respectively, as tracers for the carbon skeleton and nonamino nitrogen of amino acids supplied to cells should make possible a decision as to whether all, certain, or no amino acids are utilized directly in protein synthesis within the plant.

## VI. Residual Nitrogen

The nonprotein organic nitrogen of plant tissues which is not accounted for in the amide and amino fraction is usually termed the "residual" or "other" nitrogen. This fraction usually constitutes a considerable proportion of the nonprotein organic nitrogen and may, under some conditions, as in the roots of nitrate plants (313, 335, 360) exceed the sum of the amide and amino nitrogen. This "residual" nitrogen includes polypeptide and peptide nitrogen, the nonamino nitrogen of arginine, histidine, proline, and tryptophan, purine nitrogen, and the nitrogen of the simpler natural bases, alkaloids, glycosides, and vitamins.

Our knowledge of the role in plant metabolism of the purines, simpler natural bases, alkaloids, and glycosides is meager and advance in these fields is particularly handicapped by lack of suitable methods for their rapid analysis in plant extracts.

### A. BETAINES

Special interest attaches to the simpler natural bases in view of their universal occurrence in plants, their low molecular weight, obvious chemical relationship to the amino acids, and their possible significance in such important biological reactions as methylation. Choline, being a constituent of lecithin, is probably present in every living cell. Schulze and Trier (301) and Klein and Zeller (183), in an examination of more than 100 plants of widely different systematic origin, were able to detect free choline in all cases with the exception of the three lichens examined. The vitaminlike action of

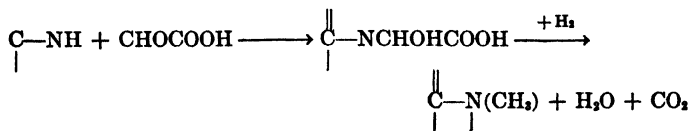
choline in animal metabolism seems to be related to its activity as a methyl donor (19,361). Klein and Linser (178,182) have studied the variations of lecithin and choline contents during the germination of seeds but were unable to draw any definite conclusions as to the physiological role of choline. There is now (324,362) direct evidence in support of the earlier suggestion of Trier (345) that aminoethanol is the precursor of choline in animal cells and the development of choline-less strains of the fungus *Neurospora crassa* (164) has enabled Jukes, Dornbush, and Oleson (172) to obtain evidence that choline is synthesized in the fungus by stepwise methylation of aminoethanol. Horowitz (163) has examined two different choline-less strains of *Neurospora*, isolated monomethylaminoethanol as an intermediate in choline synthesis, and shown that strain 34486 is unable to synthesize this intermediate from aminoethanol but can complete its methylation to choline, whereas strain 47904 cannot use even monomethylaminoethanol as a precursor for choline synthesis. Aminoethanol is known to occur in higher plants (149) but there are no data on the mechanism of its biosynthesis nor is it known to act in higher plants as the precursor of choline.

Glycine betaine was detected as early as 1906 by Stanek (319) in the leaves, roots, and seedlings of higher plants but, as with choline, its role in the metabolism of the higher plant remains obscure. Barrenscheen and von Vályi-Nagy (23) claim to have demonstrated, in wheat germ, the methylation of glycine to betaine in the presence of methionine the sulfur of which was simultaneously oxidized, but there was no evidence that glycine itself could act in methyl group formation along the lines suggested (18) from studies in animal metabolism. Stetten (323) has shown that betaine can act as a methyl donor in animal tissues and there is evidence that demethylation of betaine may be coupled with methylation of aminoethanol to choline. The biological significance of choline oxidase (223,224) is obscure. It oxidizes choline to betaine aldehyde and probably, though at a much slower rate, forms glycine betaine by further oxidation. The reverse reaction of reduction of betaine to choline has, however, not been demonstrated.

Glycine betaine is the simplest of a group of naturally occurring methylated amino acids or betaines. Trigonelline, the betaine of *N*-methylnicotinic acid and stachydrine, the betaine of *N*-dimethyl-

pyrrolidine- $\alpha$ -carboxylic acid are fairly widely distributed in plants, but rarely occur together (177). Klein and Linser (179-181) have demonstrated increased synthesis of trigonelline in their experimental plants following injections of ornithine, glutamic acid, and particularly of proline with hexamine. They regarded trigonelline as representing a store of available nitrogen. Enhanced synthesis of stachydrine was also reported to follow injection of proline. Nicotinic acid is now a well-known plant constituent and is reported as essential for the growth of excised tomato roots (394). Klein and Linser have advanced a scheme of reactions to show the possible biochemical interrelationship of glutamic acid, proline, ornithine, stachydrine, nicotinic acid, and trigonelline, but experimental support for the hypothesis is lacking and no recent work on the metabolism of betaines in higher plants is known to the author.

Evidence for primary synthesis of methyl groups in the animal cell is controversial and the mechanism of primary methyl group formation, which undoubtedly occurs in plants, is unknown. The injection of hexamine along with proline in the experiments of Klein and Linser is based on the hypothesis that formaldehyde (72,343,393) is involved in methyl group synthesis, the formaldehyde arising either as an intermediate in photosynthesis or by oxidative deamination of glycine. Schweitzer (306) obtained evidence that the polyphenol oxidase of potato oxidizes glycine with formation of formaldehyde, carbon dioxide, and ammonia. Bach (18) in a study of the mechanism of creatine formation from glycocyamine, has postulated the formation of the methyl group from glycine. It is postulated that glycine, on oxidative deamination, yields glyoxylic acid and ammonia, that the glyoxylic acid then condenses with glycocyamine, and that this product on reduction and decarboxylation, yields creatine. The formation of the methyl group would then be represented thus:



If glyoxylic acid is active in this way, methyl group formation may be connected with purine metabolism, for Brunel (57,58) has

demonstrated the presence in plants and animals of the enzyme allantoinase, which breaks down allantoinic acid into glyoxylic acid and urea. The neglect of the study of the metabolism in plants of the simple natural bases revealed by the above discussion is due to the low concentrations in which they occur in actively metabolizing cells and to the absence until recently of convenient methods for their separate estimation. The recent demonstrations of their significance in animal metabolism has stimulated development of improved analytical methods, which should now make possible parallel studies with plants.

### B. PURINES AND PYRIMIDINES

The purines should prove of particular biochemical interest since, together with the pyrimidines, they are presumably involved in nucleic acid synthesis. The purines theobromine, caffeine, 3-methylxanthine, adenine, hypoxanthine, and guanine are well-known plant constituents and uric acid, allantoin, allantoinic acid, and urea have been detected in small quantities in plants (127). The enzymes xanthine oxidase and adenase seem to be confined to animal tissues but guanase (289), uricase (57), allantoinase (128), allantoinase (57,58), and urease occur in plants. Bodansky (47) has put forward a scheme showing the enzymic interrelationships of the purines but its application to plants remains hypothetical. The work of Fosse and co-workers, however, makes it probable that the reaction sequence uric acid  $\rightarrow$  allantoin  $\rightarrow$  allantoinic acid  $\rightarrow$  glyoxylic acid and urea can occur in plants.

Robbins and Kavanagh (280) consider that the potato growth factor,  $Z_1$ , required for *Phycomyces* species is a mixture of hypoxanthine and guanine and Loofbourow (212) has shown that adenine nucleotides stimulate yeast growth. Jones and Barker (171), however, could only record inhibition of the root growth of seedlings following application of purine solutions. The routes of purine and pyrimidine synthesis and the role of these compounds in nucleic acid synthesis in plants remains a virgin field for investigation. Recent work on the purine and pyrimidine metabolism of animal cells emphasizes the difficulty of this field. Barnes and Schoenheimer (22) obtained data indicating that ammonia participates in purine synthesis and that urea, arginine, and histidine are ap-

parently unable to function as specific precursors. The identity of purine precursors was not apparent nor could data be obtained on the interrelationships of the purines and pyrimidines. Similarly Plentl and Schoenheimer (267) were unable to establish any relationship between supplied purines, pyrimidines, or creatine and nucleoprotein synthesis. The purines and pyrimidines supplied to animal cells did not appear to enter into synthetic combinations but suffered "cytoplasmic" catabolism, the purines to uric acid and allantoic acid, and the pyrimidines to urea and ammonia.

### C. ALKALOIDS

Dawson (99) has reviewed in detail the recent and extensive literature relating to the biosynthesis and physiological role of nicotine in the tobacco plant. Recent papers (86,87,168,169) also deal with the alkaloid metabolism of *Atropa belladonna* and other solanaceous plants. Nicotine is synthesized in the roots of the tobacco plant and is translocated in the xylem to the shoot. The main seat of synthesis of alkaloids in *A. belladonna* (87,168) and in *Datura stramonium* (158,184,257) is also the root and again the evidence indicates transport in the transpiration stream rather than in the phloem. There is, however, some evidence that limited alkaloid synthesis is possible in detached leaves of belladonna (86,168), and in *Nicotiana glauca* independent anabasine synthesis can occur in both root and shoot (98).

Attempts to identify intermediates in alkaloid synthesis have given contradictory results. Various workers (97,181,397) have reported that proline and nicotinic acid stimulate nicotine synthesis but Gorter (140), in a similar experiment, obtained negative results. Cromwell (87) found enhanced alkaloid synthesis in *A. belladonna* and *D. stramonium* following feeding with putrescine, arginine, hexamine, or formamol. The effect of putrescine alone or in conjunction with glucose was especially marked. This led to the suggestion (87) that putrescine was the natural precursor of hyoscyamine and on this basis a scheme of reactions was advanced to represent the biosynthesis of the alkaloid. Putrescine has been shown to arise from arginine via ornithine in certain microorganisms (131) and Srb and Horowitz (318), using arginine-less mutants of *Neurospora crassa*, have obtained evidence for the operation in plants of an ornithine cycle (192). James (168) has demonstrated the presence

of arginase in belladonna leaves and roots and obtained enhanced alkaloid synthesis in young detached belladonna leaves fed L-ornithine or L-arginine but negative results with other amino acids. The demonstration by Cromwell (87) that a slight enhancement of alkaloid synthesis often follows injection of methylamine, dimethylamine, trimethylamine, chlorine, and glycine but that betaine has no such effect may be of significance in indicating the nature of the methyl group donors active in plant metabolism.

Cromwell (85,86) has inclined to the view that alkaloids are to be regarded as waste products arising from the products of protein degradation. The apparent inability of his plants to utilize alkaloid nitrogen under conditions of nitrogen starvation and the tendency for alkaloids to be deposited in dead tissues supported this view. A similar conclusion was reached by Chatterjee (74). James (168, 169) has, however, shown that alkaloids may be degraded in detached leaves and that cells of the growing regions of the plant which are characterized by their high rate of protein synthesis have a high alkaloid content. Dawson (99) has discussed the possible physiological significance of nicotine and has postulated that, while it may play no important role in the aerial organs, it may function in the roots as a codehydrase for nitrate reduction and a stimulator of nitrate absorption.

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# CHEMISTRY AND ENZYMOLOGY OF NUCLEIC ACIDS

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## I. Introduction

One characteristic of the field of nucleic acid chemistry is the paucity of comprehensive reviews and monographs. Besides the classical contributions of Feulgen (124) and Jones (192) there is only one monograph covering the entire field, namely, the treatise by Levene and Bass, *Nucleic Acids* (241). Eighteen years have passed since its appearance and no revised edition has been prepared nor has any comparable treatise been published. This is perhaps indicative of the fact that nobody is willing to carry the tremendous burden of such a task. Thus, Levene's monograph still has to serve as a primer for the novice about to embark on a program of nucleic acid research. The historical discussion and some of the experimental data will be very helpful, but the rapid progress in the nucleic acid field in recent years has rendered the remaining sections obsolete. On the other hand, there have appeared a number of excellent review articles written by specialists with emphasis on their own contributions. Thus, Hevesy discusses nucleic acid metabolism (164), while Gulland and co-workers (137,142) are concerned mainly with the chemical problems, and the reviews of Bredereck (35,37,38) cover the preparative aspects and present a chemical discussion. To this category belong also the papers by Fischer (125), Klein (216), and Tipson (400,401). Reviews on the role of nucleoproteins and the biological significance of nucleic acids are numerous, but the best up-to-date reading is provided in two volumes covering the recent symposia on nucleic acid, held at Cambridge and at Cold Spring Harbor (306,307).

The chemistry and biology of nucleic acids are now fields of endeavor equal in importance to the polysaccharide studies, and second only, perhaps, to protein chemistry. In line with the trends indicated above, the topics to be discussed here are restricted. This paper will attempt to bring out certain problems which have not found their way into the reviewing literature or which have been

treated inadequately. Besides, an attempt at a critical evaluation of earlier results in the light of recent developments will be made. The bases, carbohydrates, nucleosides, nucleotides, and, finally, the polynucleotides will be reviewed. Discussions of the enzymes concerned with nucleic acid metabolism will be interspersed in appropriate places, and no rigid separation of topics has been attempted. Emphasis will be placed on the chemical and enzymological aspects, since a discussion of nucleoproteins and the biological problems related to nucleic acids would go beyond the limits of this review.

## II. Nomenclature

The compounds to be discussed here may be classified in two main groups: One comprises ribo- and desoxyribonucleic acids and their split products; the other, coenzyme nucleotides such as the adenine, riboflavin, and nicotinamide nucleotides. On the latter compounds several reviews have appeared recently (177,212,258,260,337,414) and the discussion of them will be restricted, therefore, to some questions which result from consideration of the polynucleotides and their structural units. The borderline between these two classes is not very sharp with respect to chemical aspects.

It has been proposed by Oppenheimer (308) to restrict the term *nucleotide* to those phosphorylated glycosides obtained by degradation of nucleic acids. This definition would include a rather limited number of compounds, and it would exclude the coenzyme nucleotides. The definition given in Beilstein's handbook of organic chemistry (23) is more liberal. It is suggested there to extend the limits from the split products of nucleic acids to closely related compounds which occur in nature and to include even nonbiological synthetic purine and pyrimidine *N*-glycosides. It would seem timely to enlarge this definition further to include riboflavin phosphate, despite the fact that its carbohydrate is D-ribitol rather than ribose, and the nicotinamide nucleotides, although the pyridinium base is not a conventional nucleic acid constituent. The combination of these compounds with adenylic acid in the coenzymes further emphasizes their relationship to nucleotides. Indeed, in most textbooks and reviews the policy to enumerate these coenzymes along with constituent nucleotides of nucleic acids has been tacitly adopted. Perhaps the most elastic definition of nucleosides would be as *N*-glycosides

of naturally occurring bases, the carbohydrate being D-ribose, D-2-desoxyribose, or some closely related sugar. Such a definition would include riboflavin, nicotinamide riboside, and even adenine thiomethylriboside, but it would leave out vicine and convicine, which have D-glucose as carbohydrate. The nucleotides may be defined then as phosphoric acid esters of the nucleosides.

The polynucleotides or nucleic acids have often been designated according to their allegedly characteristic source (318). This has led to an abundance of names for entities which still are ill-defined. A preferable usage is that of the noncommittal designations ribonucleic acid and desoxyribonucleic acid (141). These terms should be understood as collective nouns, since evidence is accumulating that each type is multiple in nature. Once the structures of nucleic acid specimens are better known, the names may be amended by reference to peculiarities in origin or make-up. The abbreviations RNA (ribose nucleic acid) or PNA (pentose nucleic acid) and DNA (desoxypentose nucleic acid) are used widely in original contributions appearing in some periodicals. They are convenient for the expert, but cumbersome for the average biochemist whose memory is taxed by an ever increasing number of abbreviations.

### III. Structural Units of Nucleic Acids

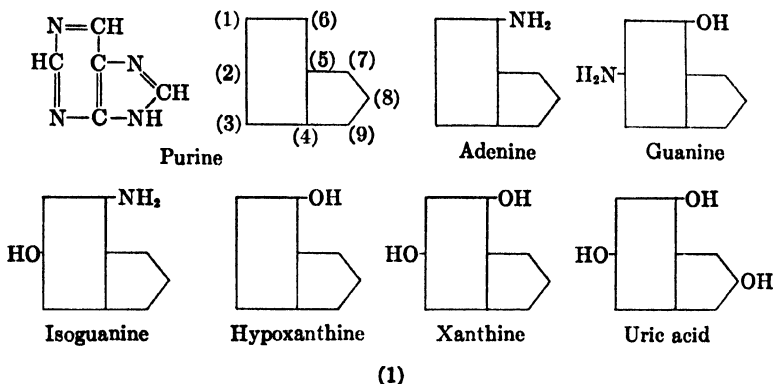
#### A. PURINE BASES

Formulas (1) represent the purine bases belonging to this group. (The author prefers the notation in these formulas to the combination of a hexagon with a pentagon. It is felt that hereby the bases of nucleosides are set apart somewhat from other heterocyclic compounds. Besides, this notation allows the convenient and self-explanatory shorthand writing in this and some of the following formulas.)

Purine itself does not seem to be a biological compound. The concentration of adenine in nature is greater than that of the other bases, since it is a constituent of several coenzymes as well as of the nucleic acids. Among the adenine compounds the adenosine-5'-phosphoric acids occur in amounts approaching those of nucleic acids in some instances, while the adenine riboflavin nucleotides and adenine nicotinamide nucleotides are found in much smaller concentration. Next in abundance to adenine is guanine, which seems to occur in

all nucleic acids. Compared to these two compounds, the other purine bases are found only in much smaller concentrations.

Hypoxanthine occurs in combination with sugar and phosphate as a precursor and as a degradation product of adenine compounds. Xanthine and uric acid hold well-established positions as intermediates in the present concept of purine metabolism. Of interest is the discovery of 2-hydroxy-6-aminopurine (isoguanine, hydroxy-



adenine) in combination with ribose in croton beans (*Tigium croton*) (67) and it remains to be seen, whether a systematic search of other source material will reveal a wider distribution of this substance. Buell and Perkins (56) were able to isolate from pig blood small amounts of a base which probably was identical with isoguanine. The method of isolation suggests that the base occurs as part of a nucleotide or nucleoside (55a). It may be that this compound is an intermediate in the transformation of adenine to guanine (see Sect. III. B.).

The occurrence of purine and pyrimidine bases in the free state in mammalian organism seems to be restricted, but there are numerous reports on isolation of purine bases from plants (424). Here, the purine bases, in contrast to the pyrimidine bases, were often found in the free state. In some instances this may have been due to inadvertent enzymic or chemical splitting in the course of the isolation procedure. It seems, however, that xanthine plays a specific role in the plant kingdom (57) as a precursor of the methylated 2,6-dihydroxypurines, notably, caffeine (1,3,7-trimethyl-2,6-dihydroxypu-

rine), theobromine (3,7-dimethyl-2,6-dihydroxypurine), theophylline (1,3-dimethyl-2,6-dioxypurine), and heteroxanthine (7-methyl-2,6-dihydroxypurine). Buchanan and co-workers (54,55) have reviewed and extended the evidence of demethylation of these compounds in the mammalian organism (26a,297a).

#### B. BIOSYNTHESIS OF PURINE BASES

Of all applications of isotope techniques in the nucleic acid field perhaps the most important results so far have been achieved in tracing the precursors and pathways of purine synthesis. Older theories assumed formation of uric acid from such units as tartronic acid and urea (325,422) or histidine and arginine (2,21,153). Several investigators had tried to arrive at conclusions by feeding diets high or low in the hypothetical structural units, but, as the extensive literature on this subject proves, the outcome of such experiments has been equivocal in most instances. At such a state of uncertainty, there was little chance for discovery of individual enzymes bringing about the synthetic steps leading to purines, particularly since the use of cell-free preparations has not been possible so far (25,103, 226,309,310) and the pathways may vary in different species (15, 101,301). The use of isotopes promises now to shed light into many phases of the problem. At first, isotope experiments merely helped to pass on the acceptability of certain compounds as precursors. The experiments of Schoenheimer and co-workers on rats with  $^{15}\text{N}$ -containing compounds eliminated urea from consideration (19), and it was found (317) that rats and pigeons cannot proceed with the pyrimidine ring to build the purine skeleton. On the other hand,  $^{15}\text{N}$  provided as ammonium citrate was readily incorporated into nucleic acid bases.

The studies of Schoenheimer have been extended recently in important contributions by Sonne, Buchanan, and Delluva (53,372), who tested the following compounds labeled with  $^{13}\text{C}$  on pigeons:  $^{13}\text{CO}_2$ ,  $\text{H}^{13}\text{COOH}$ ,  $\text{CH}_3^{13}\text{COOH}$ ,  $\text{NH}_2\text{CH}_2^{13}\text{COOH}$ ,  $\text{CH}_3\text{CHOH}-^{13}\text{COOH}$ , and  $^{13}\text{CH}_3^{13}\text{CHOHCOOH}$ . The uric acid excreted by the birds was collected and the location of the isotope studied by stepwise degradation according to conventional methods (102,357-359) and analysis in the mass spectrometer. It was found that the carbon of formate, the carboxyl carbon of acetate, and the  $\alpha$ - or  $\beta$ -carbon atoms of lactate participate in the ureide synthesis (carbon atoms 2

and 8) but that carbon dioxide, glycine, and carboxyl-labeled lactic acid could not be traced in these positions (see Table I). The concentration of  $^{13}\text{C}$  was almost identical in both rings, and it is suggested that reactions leading to the ureides are the same in both instances. There seems to be some species specificity, since the authors (53,372) observed failure of the rat to utilize directly the carboxyl carbon of acetate, in contrast to the experiments with pigeons cited above. Further experiments with pigeons gave informa-

TABLE I  
PRECURSORS OF UREIDE CARBON ATOMS AND CARBON CHAIN OF URIC ACID  
IN PIGEON\*

Expt. no.	Precursor	$^{13}\text{C}$ concentration, atoms per cent excess							
		Labeled carbons	Res- pira- tory $\text{CO}_2$	Uric acid carbon no.					
				2	4 + 5	4	5	6	8
1	$^{13}\text{CO}_2$	8.13	0.28	0.02	0.04	0.07	0.00	0.25	0.02
2	$\text{H}^{13}\text{COOH}$	3.34	0.01	2.41	0.08	—	—	0.01	2.41
3	$\text{CH}_3^{13}\text{COOH}$	5.82	0.26	2.02	0.04	0.07	0.00	0.22	2.10
4	$\text{NH}_2\text{CH}_2^{13}\text{COOH}$	5.20	0.12	0.00	—	1.16	0.14	0.11	0.00
5	$\text{DL-CH}_2\text{CHOH}^{13}\text{COOH}$	8.80	0.25	0.01	—	0.37	0.00	0.26	0.01
6	$\text{DL-}^{13}\text{CH}_2^{13}\text{CHOHCOOH}$	5.40	0.11	0.10	—	0.07	0.14	0.09	0.10

\* Data of Buchanan, Sonne, and Delluva (53).

tion about the synthesis of the carbon chain comprising atoms 4, 5, and 6 in uric acid. The isotope-containing precursors and the location of the isotopic carbon are given in Table I. Carbon dioxide is utilized for position 6, while formate does not enter in this place. The authors believe that this may perhaps indicate a new carbon dioxide assimilation reaction. Carboxyl carbon of glycine is placed in position 4, and the amino nitrogen of this compound seems to furnish nitrogen atom 7, as indicated by the use of  $^{15}\text{N}$ -labeled glycine (1,362). In the experiments reported in Table I the respiratory carbon dioxide was measured by keeping the animals in a closed system. Its determination permits a correction for oxidation of the administered substances.

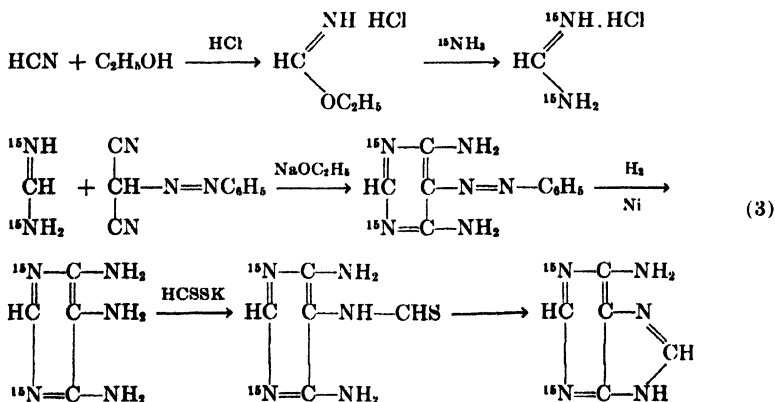
This work with isotopes may be considered a foundation for important future work (135a). So far it does not reveal much about the enzymes bringing about purine synthesis, but reference should be made here to the role attributed to *p*-aminobenzoic acid and folic



acid in this process (233,330,365; see also Sect. III. E.). The isolation of intermediates of purine synthesis is particularly important. One of them has been observed by Stetten and Fox (382) to accumulate in a sulfonamide-containing bacterial medium. Shive and co-workers (321,363) were able to identify it with 5(4)-amino-4(5)-imidazolecarboxamide (423) which has the structure given in formula (2). In this compound only one carbon atom of the purine system is missing.



Another recent example of successful use of isotope-containing nucleic acid constituents is provided by the admirable work of Brown, Roll, Plentl, and Cavalieri (50,51). Adenine, labeled with  $^{15}\text{N}$  in positions 1 and 3, was synthesized by Traube's method as modified by Baddiley, Lythgoe, and Todd (14,209). The method is given in formulas (3). The base was fed to rats at a level of 250 mg. per kilogram of body weight, which proved to be below the toxicity level during the three-day period of experimentation. The animals were sacrificed and nucleic acid was isolated from the dehydrated viscera. Hydrolysis and fractionation yielded adenine and guanine, which



were carefully separated. Another fraction contained the pyrimidines, and adenosine triphosphate (ATP) was isolated from the leg and back muscles. From the urine during the experimental period, allantoin, urea, and ammonia could be isolated and examined. It was found that 13.7% of the adenine in the nucleic acids was incorporated from the labeled dietary supply, and 8.2% of the guanine nitrogen was derived from the labeled adenine. No labeled nitrogen had found its way into the pyrimidine fraction, and little was present in the adenosine-5'-phosphate fraction. Of the urinary constituents, allantoin contained the largest part of the isotopic nitrogen while little was found in urea and ammonia. From these results, Brown and colleagues were able to draw the following conclusions: The rejuvenation of adenine in the nucleic acids is considerable, but it remains small in the adenosine triphosphate fraction. Adenosine-5'-phosphate, therefore, does not seem to be an intermediate in the formation of the adenylic acid (adenosine-3'-phosphate) present in ribonucleic acid. Dietary adenine can be converted to guanine but not to pyrimidines. The decomposition of the purine nucleus yields predominantly allantoin, which is in line with earlier concepts. It is important that urea and ammonia nitrogen in the urine were not derived from nitrogen atoms 1 and 3 of the adenine.

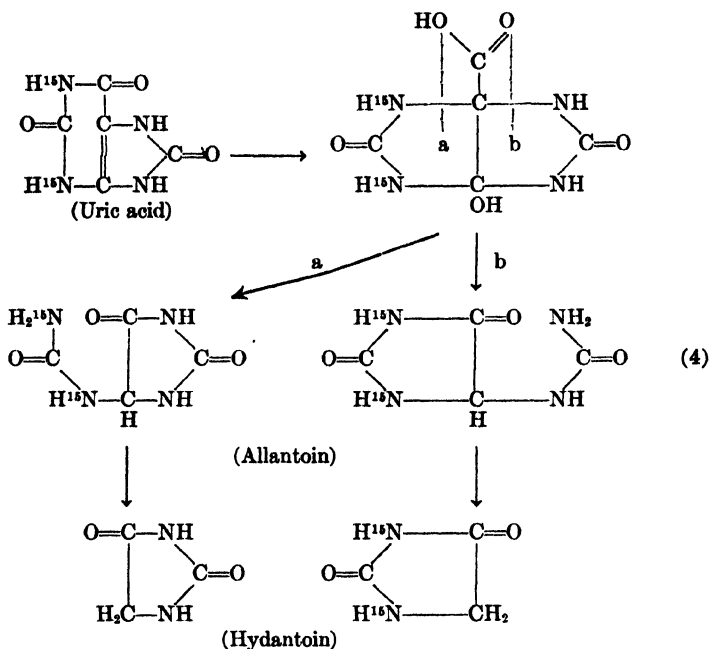
Corresponding experiments with guanine (51) confirmed the earlier observation of Plentl and Schoenheimer (317) that dietary guanine is not incorporated into nucleic acids. Guanine can be converted, however, to allantoin, and its 2-amino group when labeled can be traced in the urinary ammonia and urea. This is due to the action of guanase.

More recently it was found that labeled dietary 2,6-diaminopurine (2-aminoadenine) is also converted into guanine by the rat (23a). The occurrence of this compound in nature has not yet been demonstrated, but it may be an intermediate in the conversion of adenine into guanine.

### C. BIOLOGICAL DEGRADATION OF URIC ACID

While man and other primates excrete uric acid as a terminal product of purine metabolism, with very few exceptions all other mammals eliminate allantoin. The decomposition of uric acid to allantoin by the enzyme uricase has been the subject of many studies. Felix and co-workers (122) claimed a multiple nature of

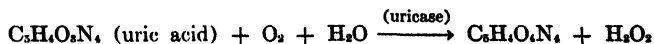
uricase, and, particularly from the work of Schuler and Reindel (322,323,357-359), it has become clear that the degradation (50) occurs in several steps (formulas 4). As an intermediate, hydroxy-



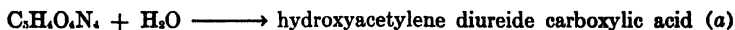
acetylene diureide carboxylic acid was postulated. Its decarboxylation and splitting would lead to allantoin. However, general agreement concerning the nature of uricase and the intermediates was not reached (80,207,220,405). In favor of hydroxyacetylene diureide carboxylic acid as an intermediate was the observation that racemic allantoin, instead of the optically active modification, is isolated from urine (358). The experiments with labeled purines cited in the preceding section permitted a reinvestigation of this problem (49,50) based on the following considerations: The nitrogen atoms in the imidazole rings of hydroxyacetylene diureid carboxylic acid are derived from the 1 and 3 positions of the purine nucleus and the 7 and 9 positions, respectively (see formulas 4). Use of a 1,3-labeled purine will yield the compound with isotopic nitrogen in only one

ring. If the symmetrical compound actually is an intermediate, the enzyme which opens one of the rings to form allantoin will not distinguish between the imidazole rings. Therefore, with equal chances that splitting occur according to *a* or *b* in formulas (4), half the isotopic nitrogen should be found in the ring of allantoin, the other half in the ureido group. The formation of hydroxyacetylene diureide carboxylic acid as an intermediate would then be verified. For analysis, reductive degradation to hydantoin was carried out. Had all  $^{15}\text{N}$  been concentrated in the ureido group of allantoin, the hydantoin would have been found free from an excess of isotope, and conversely, with the isotope located exclusively in the imidazole ring of allantoin, the resulting hydantoin should have contained all of it. The experiment showed that in accordance with formulas (4) half the isotope of allantoin was present in hydantoin. This result proves the intermediate formation of hydroxyacetylene diureide carboxylic acid or a like symmetrical intermediate (62a,b).

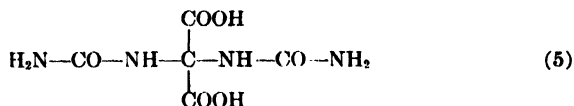
The enzyme uricase, which brings about the degradation of uric acid, deserves further study. According to recent reports it contains zinc (80,181,411). Klemperer has reinvestigated earlier concepts of uricolysis (220). He came to the conclusion that allantoin cannot be the only product of uricase action, since the amount of carbon dioxide formed was only a fraction of one equivalent for every molecule of uric acid which had disappeared. The following mechanism (220) was suggested: The first step is the formation of a very labile and as yet unidentified product of oxidation:



The intermediate then is assumed to undergo decomposition according to one of the following alternatives:

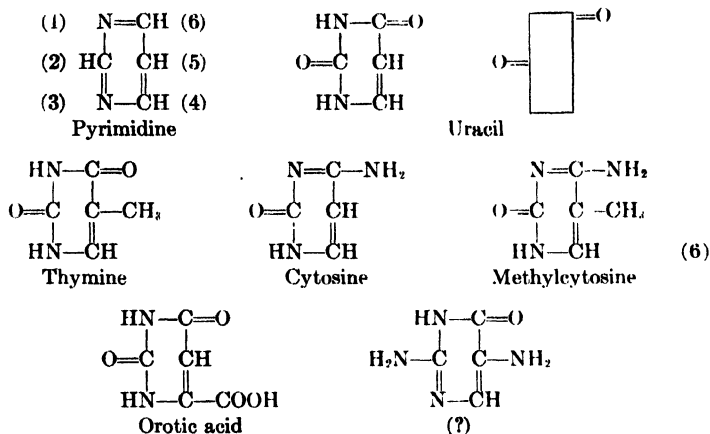


The structure of uroxanic acid is shown in formula (5). Reactions *a*, *b*, and *c* in all probability are spontaneous chemical decompositions, and the relative amounts of reaction products depend on factors like *pH*, temperature, and the type of ions present.



## D. PYRIMIDINE BASES

The pyrimidine bases of significance in nucleic acid chemistry are represented in formulas (6). Cytosine is a part of both ribonucleic acids and desoxyribonucleic acids, while uracil seems to be

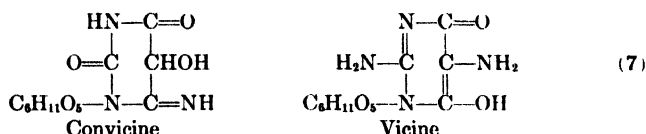


restricted to the former, and thymine to the latter class of compounds. The occurrence of 5-methylcytosine in tuberculinic acid, the nucleic acid from *Mycobacterium tuberculosis*, has been reported by Johnson and Coghill (189). The criteria of identification, however, remained limited, since very little material was available. Recent experiments of Hotchkiss (182) indicate its presence in small amounts in desoxyribonucleic acid from thymus gland. Notwithstanding the difficulties of repeating the isolation, it would seem most desirable to confirm the occurrence of this base (409a). The same holds true for a diaminohydroxypyrimidine, whose isolation in small amounts from some yeast nucleic acid preparations has been reported by Kutscher (232). Johnson has suggested the identity of the base with 2,5-diamino-6-hydroxypyrimidine (190). Apparently, it has not been encountered by any other investigators,

which shows that its occurrence in nucleic acid hydrolyzates is rare, if not fortuitous (409a).

The significance of orotic acid, which was first isolated from skim milk by Biscaro and Belloni (29), has been the subject of much speculation (13,167,188,420). Johnson suggested that it may be a key substance in pyrimidine metabolism (187), but Mitchell, on the basis of his work with *Neurospora* mutants, considers orotic acid a by-product rather than an intermediate of nucleic acid biosynthesis (292). Recent experiments of Hammarsten and his colleagues (25a) may settle this question. Orotic acid containing  $^{15}\text{N}$  was injected into rats. From the liver polynucleotide fraction, labeled cytidine and uridine were isolated. This proves utilization of orotic acid in the biosynthesis of both uracil and cytosine.

Noteworthy in this connection are two pyrimidine glycosides, vicine and convicine, which have been isolated (424) from the seeds of *Vicia sativa* and *Vicia faba* (sweet pea). The bases are divicine (2,5-diamino-4,6-dihydroxypyrimidine) and 4-iminodialuric acid (129). For some time the compounds were considered to be nucleosides, but in later investigations the sugar proved to be D-glucose (238,425), and therefore vicine and convicine (see formulas 7) can



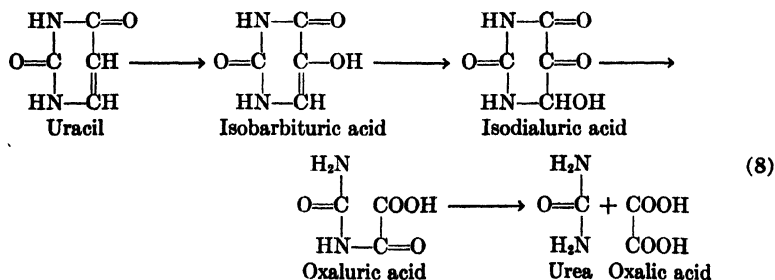
no longer be classified as members of this group. They deserve attention, however, as close relatives of the pyrimidine nucleosides, and they provide a link between this class and other *N*-glycosides.

#### E. PYRIMIDINE SYNTHESIS AND DEGRADATION

Higher animals can synthesize pyrimidines under normal conditions. Dietary pyrimidines are poorly utilized according to Plentl and Schoenheimer (317). They are metabolized directly without prior incorporation into the nucleic acids. It may be opportune to point out here that the results of experiments on incorporation of labeled dietary purine and pyrimidine bases are not sufficient to develop a complete picture of the events under normal dietary con-

ditions. In the intestinal tract nucleic acids are broken down to the nucleosides and absorbed in this form. The free bases must be considered unusual dietary constituents. Elimination of labeled fragments in the urine proves that the free bases too are absorbed, but it appears possible that dietary nucleosides offer a better chance of utilization in anabolic processes. Unfortunately, the procurement of sufficient amounts of labeled nucleosides is difficult. While labeled purines and pyrimidines can be synthesized with comparative ease by the organic chemist, there is no chance at present of getting labeled nucleosides in quantity by routes other than biosynthesis employing yeast or bacteria.

For the synthesis of pyrimidines, amino acids can furnish the building material, but the individual steps in the process are still obscure. Stokes (387) has reported that folic acid functions in the bacteria as a coenzyme for the enzyme system which is responsible for the synthesis of thymine or a thymine-like compound. Support of this view has come from the experiments of Shive and of Spies (321,330,375,377), but others (156) have questioned Stokes' concept, and Hitchings (172,173,175) in particular favors restraint in the interpretation of Stokes' and of Shive's experiments. Very recently, Shive and his colleagues have been able to demonstrate that thymidine is effective in counteracting the growth inhibition of *Leuconostoc mesenteroides* by sulfanilamide compounds (364). This report was followed immediately by the demonstration that thy-



midine is an essential growth factor for several lactobacilli (370,427; see also Sect. V. C.).

Thanks to the investigations of Cerecedo (63,110,379) we have a satisfactory picture of pyrimidine degradation in the mammal.

The phases of it are exemplified with the degradation of uracil in formulas (8). Urea and oxalic acid are urinary end products. Dietary cytosine and methylcytosine were found partly unchanged in the urine, but in a natural diet the former is most likely combined with pentose while the latter is of doubtful biological significance in the mammal. Dietary cytidine is converted to urea and the ribose does not appear in the urine (110).

#### F. ANALYTICAL DETERMINATION OF NUCLEOTIDE BASES

Important strides have been made recently in the analytical techniques of purine and pyrimidine determination. The estimation of these bases after hydrolysis has often been taken as a measure of nucleic acid concentration in tissues (195,346), but the task is cumbersome and losses are unavoidable. For nucleic acid determination the isolation of the bases has largely been superseded by the colorimetric carbohydrate tests (see Sect. III. M.). The importance of quantitative determination of the bases has shifted to the search for deviations from their uniform distribution in nucleic acids (see Sect. VII. H.).

Conventional chromatography has been used very little in nucleic acid chemistry, but paper chromatography seems to offer much promise for purine and pyrimidine analysis (64a,182,409). Revolutionary are the changes brought about by adaption of ultraviolet spectrophotometry. Its possibilities are given in Section IV. The countercurrent distribution procedure of Craig (77) has become another very valuable tool in purine and pyrimidine analysis (399). Surprisingly, microbiological assay has not found as wide application as one might expect. Numerous bacteria are known which, due to the ample supply of purines and pyrimidines in their natural habitat, have lost the ability to synthesize the bases and, therefore, are dependent on an outside supply for nucleic acid synthesis and growth. One of the obstacles in using microorganisms for purine and pyrimidine assay is their lack of fastidiousness with respect to the type of base utilized. It seems that the ability to interconvert the purines and pyrimidines according to need has not been lost by the bacteria in most instances, and often the bases serve as growth stimulants rather than essentials (222,227,294,329,369,371,426). The field is barely touched, however, and, no doubt, elaboration of microbiological methods analogous to the techniques of vitamin determi-



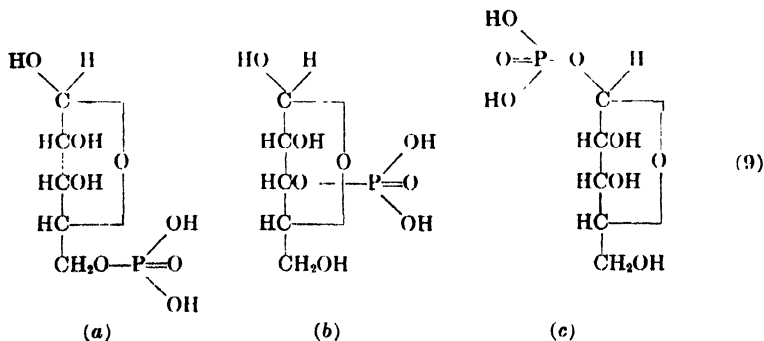
nation will be forthcoming (22a). *Neurospora* mutants are already used for this purpose (see Sect. VII. H.).

### G. CARBOHYDRATES

The only carbohydrates which have so far been established as constituents of nucleic acids are D-ribose and D-2-deoxyribose. The closest relatives occurring in compounds of this class are thiomethylpentose, isolated from adenine thiomethylpentoside, and D-ribitol, which in combination with alloxazine is a constituent of the riboflavin nucleotides. The structure and metabolism of these carbohydrates will be discussed in the following pages.

### H. RIBOSE AND ITS PHOSPHORIC ACID ESTERS

The nature of the carbohydrate from pentose nucleic acids was established around 1910 by Levene and co-workers (241). It remained generally accepted until 1927 when Robinson questioned the occurrence of ribose in the nucleosides and nucleotides (326). He believed that the hydrolysis in the isolation process causes a Walden inversion which would yield ribose from another sugar, but this theory was speedily disproved by Levene (240), and the isolation of this carbohydrate from various nucleosides under different conditions of hydrolysis corroborated Levene's contention (67,336,374). Only in recent years has the occurrence of pentoses other than D-ribose in pentose nucleic acids been seriously considered again. Gulland and associates reported the isolation of small amounts of L-lyxose and D-arabinose from yeast nucleic acid hydrolyzates (140). The significance attributed to this finding was soon discredited by



Gulland himself, who came to the conclusion that these sugars represent artifacts derived from ribose by epimerization in the course of isolation (18).

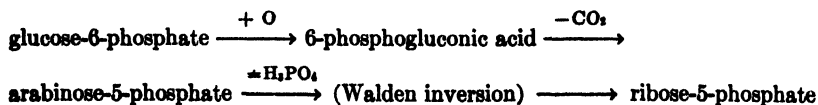
The ribose phosphates described so far are D-ribose-5-phosphate, D-ribose-3-phosphate, and D-ribose-1-phosphate (see formulas 9, a, b, and c, respectively). The evidence for the structure of the former two has been summarized by Levene (241,242,251). Ribose-1-phosphate has been discovered recently by Kalckar (201). Although it has not been isolated in pure form, its constitution as proposed by this author seems well-founded because of its origin from nucleosides. It was observed that nucleosidase is a phosphorolytic rather than a hydrolytic enzyme and, since splitting occurs at carbon atom 1 of the sugar, formation of ribose-1-phosphate seems indicated. The isolation is complicated by the great lability of the ester. In 0.5 *N* sulfuric acid at room temperature Kalckar found the half-time of splitting to be 2.5 minutes, which shows that the ester is somewhat more acid-labile than phosphocreatine, but less sensitive than acetyl phosphate (201). Hydrolysis released about one equivalent of aldose for each molecule of labile phosphoric acid liberated. The compound thus has several features in common with glucose-1-phosphate, although it is much more labile. Confirmation of the structure as proposed by Kalckar and procurement of larger amounts would seem to make a chemical synthesis most desirable. The ribose derivatives necessary as intermediates, however, are rather difficult to handle.

#### I. ORIGIN AND METABOLISM OF RIBOSE

Until recently little information about the metabolic fate of ribose was available, and the problem of its origin was subject to considerable speculation (137). Elvehjem pondered the classification of ribose as an essential dietary constituent for mammals (108). Gardner (135) suggested a combination of glycol aldehyde with triose to form pentose, which seemed possible in view of observations on aldolase by Meyerhof and Lohmann (261,284,395). These authors stated that triose phosphate and glycol aldehyde can be converted by aldolase into pentose, but experimental details were not reported. Progress in relation to these questions was hampered, since the free sugar is either not metabolized by tissue preparations or yeast, or at most at a much slower rate than the ordinary hexoses (87,198,

430). On the other hand, ribose phosphates or nucleosides were rare chemicals until recently and few biochemists were in a position to use them extensively. Even experimentation with ribose was costly in earlier years. Larson and co-workers (234) have reported that ribose administered to albino rats by mouth is poorly utilized, while intraperitoneal injection gives some increase of liver glycogen. Poor absorption from the alimentary tract was also observed by Wuest and Solmssen (428) and by Naito (299), which is in line with the variations of the absorption factor reported on monosaccharides by Cori (75).

In the course of studies on enzymes concerned with nucleoside metabolism, Lutwak-Mann observed that, after incubation of adenosine with *Escherichia coli* until deamination and nucleosidase action had come to completion, no ribose could be discovered in the medium (273). This observation was followed by a more extensive study by Stephenson and Trim (380), who confirmed the earlier results but did not identify any reaction products. From Dickens' laboratory (86-88) came significant contributions to the subject in an investigation on the oxidation and decarboxylation of glucose-6-phosphate, which were discovered by Lipmann (257) and by Warburg (416). Arabinose-5-phosphate was expected as an intermediate of glucose-6-phosphate degradation, but the synthetic pentose ester was found rather inert toward yeast enzymes. Therefore, Dickens examined other pentose phosphates and found ribose-5-phosphate to react with a speed justifying its assumption as an intermediate in the process. A Walden inversion was suggested to account for the appearance of ribose phosphate instead of the expected arabinose phosphate, according to the scheme:



The decomposition of ribose phosphate by yeast enzymes follows the equation (87):

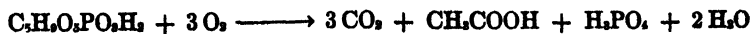


The two carbon compound in this equation could not be identified.

The knowledge of ribose metabolism was furthered also by Dische

(91), who reported that incubation of adenosine with hemolyzate causes disappearance of the pentose from the reaction mixture. Colorimetric tests and hydrolytic experiments indicated the formation of hexose diphosphate (94). Waldvogel and Schlenk observed that incubation of adenosine with tissue preparations results in disappearance of the pentose from the sample with simultaneous esterification of phosphate (340). The observation could be extended to a large number of nucleosides and nucleotides (341) and the enzymes necessary were found mainly in those tissues which are particularly active in nucleotide metabolism. The esterification of phosphate seemed to be explained by the results of Kalckar, who had demonstrated the formation of ribose-1-phosphate by nucleosidase action (201) but, in contrast, to the lability of Kalckar's ester, the phosphoric acid was bound very firmly in the experiments cited above. This indicated a rearrangement of ribose-1-phosphate into a stable compound. Ribose-3- and -5-phosphates were considered as possible intermediates in the process. Only the latter reacted while the former was found inert. It seems possible (340) that ribose-5-phosphate is formed from ribose-1-phosphate by a rearrangement analogous to phosphoglucomutase action (73,300). The steps in the degradation of ribose remain to be elucidated, but it is probable that the tissue enzymes act like yeast enzymes, the first step being aldolase action, yielding triose phosphate and perhaps glycol aldehyde (304). From the phosphoric acid ester mixture present after incubation, Robison ester could be isolated in good yield (412). This accounts for the triose, but glycol aldehyde was not found. One may assume that it is metabolized rapidly and not accumulated. It is apparent from these experiments that in the mammalian organism at least three of the five carbon atoms of ribose are derived from triose, and a bridge to hexose metabolism is thus established.

Similar pathways of synthesis and degradation of ribose seem to exist in bacteria. Krampitz and Werkman found (224) that *Staphylococcus aureus* metabolizes the ribose from nucleic acid and nucleosides according to the equation:



Here again an aldolase split seems to occur; the triose is completely

oxidized while the two carbon compound appears as acetic acid. It is probable that the latter is formed from glycol aldehyde by an enzymic inter- or intramolecular rearrangement. Very recently the study of pentose phosphate formation and breakdown in bacteria has been extended by Racker (320). Triose phosphate was found to be an intermediate and it was possible to accomplish condensation with glycol aldehyde in the presence of aldolase. The resulting pentose phosphate, however, was not identified. Since several groups of investigators are now working on this problem, one may anticipate rapid advances in the near future.

#### J. DESOXYRIBOSE

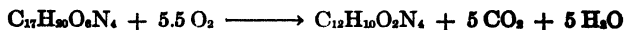
Desoxyribose was mistaken by early workers for a hexose, and the literature prior to 1930 should be interpreted with this in mind. Feulgen was probably the first to realize the unusual nature of the sugar of thymonucleic acid (123), but it remained for Levene, London, and Mori (246-248) to establish its identity with D-2-desoxyribose.

There is no information on the biosynthesis and metabolism of desoxyribose, but the recent developments in the field of ribose metabolism have caused considerable speculation. There is an indication that phosphorylated intermediates play a role, since Klein has shown that phosphate ion is required for nucleosidase action on desoxyribose nucleosides (215). A phosphoric acid ester of desoxyribose has been described very recently (281).

#### K. RIBITOL

Although alloxazine nucleotides are not included in this review, a brief consideration of the carbohydrate group of riboflavin is included here for comparative purposes. D-Ribitol has been established as a constituent by synthesis (206,228), which also established the location of the phosphoric acid in the riboflavin coenzymes in position 5 of the ribitol (229). Isomers of riboflavin containing other pentitols have been prepared and their inferiority as vitamin B<sub>2</sub> illustrates the special role of ribose derivatives in nature (114,229). The fate of the carbohydrate moiety in photolysis of riboflavin has not been investigated and no nucleosidaselike enzyme for this compound has been described. Foster has described (133) an organism,

*Pseudomonas riboflavinus*, which oxidizes riboflavin to lumichrome according to the equation:



#### L. THIOMETHYLPENTOSE

Hydrolysis of the adenine- and sulfur-containing nucleoside from yeast gave a thiomethylpentose, which was investigated by Suzuki and Mori (390) and by Levene (250). The Japanese authors came to the conclusion that the sugar is a 5-thiomethylpentose, while Levene favored the allocation of the thiomethyl group to position 3. Wendt (419) has extended the work and confirmed the configuration proposed by Suzuki and Mori. The assumption that the compound is a derivative of ribose rather than of any other pentose is arbitrary, and no evidence of interconversion of this sugar with other pentoses in nature has been obtained so far (342).

#### M. COLORIMETRIC PENTOSE TESTS

Carbohydrate color tests have been used for many years to detect and determine nucleic acids and their derivatives. Only in recent years, however, have they found wide application. This is due to a number of developments, among them the increased interest of investigators in nucleic acid determinations in a large variety of materials, the discovery of simple techniques which often obviate the necessity of separation of the nucleic acid from other cellular constituents, and the development of simple and reliable photoelectric measuring instruments. The amounts needed are, as a rule, very small and the estimation of the carbohydrate group of nucleic acid does not require more time than the colorimetric phosphate determination. The accuracy, however, is not the same as that of the latter method.

The tests for ribonucleic acid and ribose-containing split products are based on the formation of furfural upon treatment with hot acid. Older methods involved a cumbersome distillation of the aldehyde prior to its determination. This has been abandoned in favor of coupling the furfural with a color-yielding phenol, usually orcinol, in the reaction mixture which saves much time and material.

Extensive investigations on colorimetric carbohydrate tests have been carried out by Dische (90,95), and great interest in pentose determinations was initiated by Mejbaum's adaptation of the orcinol reaction of Bial (283). This procedure requires less than 0.1 micromole of ribose or ribose-containing material, which is dissolved in hydrochloric acid containing orcinol and a small amount of ferric ion as catalyst. Heating at 100° splits the nucleic acid or related pentose-containing material, converts the sugar into furfural, which reacts immediately with the orcinol to give an intense and stable green color

(see Fig. 1). Many laboratories have introduced minor modifications to suit their special needs. The following points have found consideration: The acidity has to be equal in the unknown and the reference samples, because the color depends greatly on the acid concentration (211). A heating time longer than that recommended by Mejbaum is advocated (7,336,341) and the amount and type of catalyst has been changed by some investigators (20,211, 282). As a reference standard, any pentose may be used under the experimental conditions given by Mejbaum, but the color response in some of the modified procedures varies with the material tested (20). It is advisable, therefore, to take a standard identical with the unknown material, or one which closely resembles it. Orcinol is the most widely used phenolic reagent, but phloro-

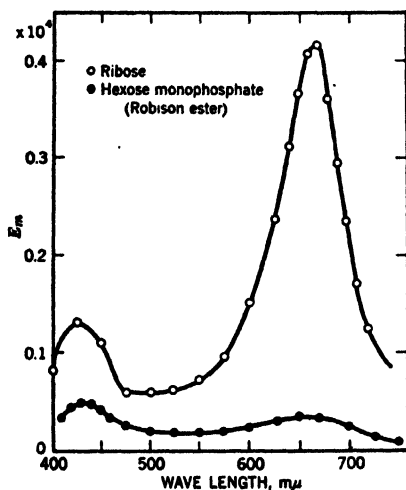


Fig. 1. Absorption spectrum of pigment obtained in the orcinol pentose test (413).

glucinol or *p*-bromoaniline is preferred by some laboratories (112,328). Riboflavin and thiomethylpentose do not react (413) under the experimental conditions specified by Mejbaum (283). Ketoxylase reacts like about 0.8 equivalent of aldopentose (161), and some response is given also by potential sources of pentose, like 2-ketogluconic acid, 5-ketogluconic acid (26,93,429), and ketoheptose (327). Protein does not interfere very much and it is apparent from Figure 1 that hexoses do not give a colored product of comparable extinction at 670 mμ (47). The specificity of the test can be further increased by extraction of the green pigment with amyl or butyl alcohol (278,286,341). Some investigators have attempted to distinguish between free pentose and various pentose phosphoric acid esters and glycosides by variation of the heating time and acid concentration (7,95,328). It should be emphasized that

great caution is necessary in the interpretation of results based on different rates of color development.

The determination of desoxyribonucleic acid is carried out mostly by Dische's method (90). The sample is heated with a mixture of glacial acetic and sulfuric acids containing diphenylamine. Intensity of the purple-to-violet color corresponds to the amount of desoxyribose. Stacey *et al.* noted that  $\omega$ -hydroxylevulinic aldehyde is an intermediate in this process (377a). The test is not as sensitive as the orcinol reaction for pentoses; hence somewhat larger amounts of material are required. Protein enhances the color intensity (112), but the most serious drawback is the scarcity of suitable reference material. Desoxyribose is still one of the rare sugars and the situation is not much better with respect to desoxyribose nucleosides and nucleotides. Thus, desoxyribonucleic acid samples are usually employed as a standard, but unfortunately only in rare instances do the authors of papers report adequate analytical characterization of their reference material. The data on quantitative determination of desoxyribonucleic acid in various sources are not too accurate in many instances.

The chemistry of the diphenylamine test would seem to deserve further study. Since the sugar has not actually been isolated from all the constituent nucleotides of desoxyribonucleic acid, its identity with D-2-desoxyribose throughout the polynucleotide has been inferred merely by such criteria as positive color tests (8,28,89). Some measure of confirmation will come from increased knowledge of the specificity of these tests. Kent, Stacey, and Wiggins made the encouraging observation that the diphenylamine test is apparently specific for 2-desoxypentoses. A sample of synthetic 3-desoxyxylose failed to respond to Dische's test (210). Reference should be made to a new method of Cohen (68) using tryptophan, and another test of Dische (92,388a) using cysteine as color-producing agent. The reaction of Feulgen has retained its popularity for cytological work (79).

Analytical examination of highly purified preparations by the colorimetric carbohydrate tests is a relatively simple matter. The majority of reports is concerned with quantitative or semiquantitative nucleic acid determination in biological material. To increase the accuracy, preliminary treatment of the sample is often required. Of the methods elaborated for this purpose, the procedures of Schneider constitute a particularly significant advance (353). The comparative merits of the colorimetric tests have been reviewed recently by this author (354), and further experiences are reported in an ever increasing number of papers from various laboratories (378a).

## N. PHOSPHORIC ACID

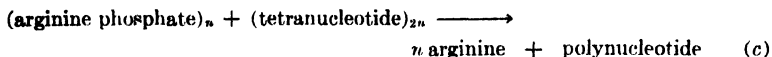
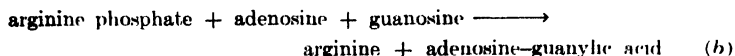
The acid constituent of nucleotides has found interest mainly with respect to the mode of its linkage and its titration behavior. It is unfortunate that single or double esterification and appearance in the metaphosphate or pyrophosphate form are not accompanied by characteristic changes in the absorption spectrum in the readily



measurable ultraviolet region. Esterification with carbohydrate as a rule renders the remaining dissociable groups more acid (285), and this should be taken into account when the construction of theoretical titration curves of hypothetical nucleic acid molecules is attempted. The stability of the phosphoric acid ester linkage depends considerably on the nature of other substituents of the sugar molecule. The bases, for example, though remote from the phosphate molecule, influence the course of its hydrolysis by acid. Thus, adenosine-3'-phosphate, guanosine-3'-phosphate, and cytidine-3'-phosphate are dephosphorylated by 0.1 *N* hydrochloric acid at 100° after thirty minutes to the extent of 39.9, 26.4, and 6.4% (244). It may be assumed that multiple esterification in the polynucleotides causes further variation in the stability of the phosphoric acid ester linkages. Obviously then the determination of rates of hydrolysis will never become as important as in the coenzyme nucleotide field in which the pyrophosphate bond provides a characteristic feature.

Recent work of Wiame (421) and others (349) has caused a revival of interest in the occurrence of metaphosphate in nature (256, 277). No direct relation of this fraction to nucleic acids has been established so far, but it is tempting to credit the highly polymerized metaphosphate with supplying orthophosphate for nucleic acid synthesis.

The nucleoside phosphate bonds are low in energy according to Lipmann's classification (258). The theories on the mechanism of the esterification are not beyond the stage of speculation. Stern (381) has suggested phosphoarginine as phosphate donor according to the following equations:



In favor of this assumption Stern mentions that the protein component of many deoxyribose nucleoproteins is rich in arginine, which in addition, is arranged in the polypeptide chains so that the transfer of phosphate would be facilitated by the spatial relationship. Several objections to this theory are obvious, however. First of all, phosphoarginine has not been established to exist or play a role except in the invertebrate organisms, which restricts unduly its significance for nucleic acid synthesis. Moreover, arginine phosphate has not been shown to exist in polypeptide form. Despite these drawbacks

the role of energy-rich phosphate compounds in this field as suggested by Stern (381) would seem to deserve serious consideration.

Studies on the mechanism of nucleic acid formation are handicapped so far by the necessity to work with intact organisms. Aside from certain disadvantages of *in vivo* experiments we face here the problem that biosynthesis and metabolism of ribo- and desoxyribonucleic acids usually cannot be separated. In view of this, the studies of Cohen (69) on the desoxyribonucleic acid of the bacteriophages  $T_2r^+$  and  $T_4r^+$  deserve particular attention. Using radioactive phosphate, Cohen found that the phosphorylated virus constituents, amounting to 40% desoxyribonucleic acid, are synthesized essentially from phosphate, which is assimilated from the medium after infection. The ribonucleic acid of the host organism has a very low turnover rate and is not a precursor of the virus desoxyribonucleic acid. These observations are in striking contrast to the concept of interrelation developed by earlier workers who claimed that protein and desoxyribonucleic acid synthesis in many types of cells are accompanied by a vigorous ribonucleic acid metabolism (32,301); for example, in the early stages of cleavage of the fertilized sea urchin egg, a conversion of ribonucleic acid to desoxyribonucleic acid has been assumed (33,61,350). None of these phenomena was observed by Cohen in the formation of bacteriophage. It appears, therefore, that either a reinterpretation of the earlier observations is necessary, or that widely diverse mechanisms of nucleic acid synthesis exist in nature. The experiments with bacteriophage are of special interest because in this system it appears possible to study the precursors of desoxyribonucleic acid uncomplicated by the metabolism of ribonucleic acid (69,319b).

The phosphorus isotope  $^{32}P$  in the form of phosphate has been used extensively by Hevesy, von Euler, and their co-workers (3,113,159) to study the turnover of nucleic acids in various biological materials. The results have been reported in numerous publications reviewed recently by Hevesy (163). The rates of rejuvenation are considerable, and since phosphoric acid brings about the internucleotide linkage in nucleic acids, one may take the results as an indication that the molecules have undergone a process of reassembly. It may not be justified, however, to refer to this as rejuvenation of the entire molecule. The nucleoside moieties apparently are not catabolized, but used by the organism to make other nucleic acid molecules,

which may be located in the place of the old one or elsewhere in the body. Brown and co-workers (48) have found the rate of rejuvenation as determined with labeled bases much slower than is apparent from experiments with labeled phosphate (52,81,159). These authors suggest that an exchange of the phosphate may take place without affecting the macromolecule of nucleic acid in every instance (48).

#### IV. Spectrophotometric Studies on Nucleic Acids and Nucleic Acid Constituents

The characteristic ultraviolet absorption of nucleosides and nucleotides is due almost exclusively to the double bonds of the nitrogenous bases which constitute a resonating system (45,46). Among the various substituents of purine and pyrimidine bases, hydroxyl and amino groups influence the position of the absorption maxima. The carbohydrate part (105,316) and phosphoric acid (302) do not exhibit significant absorption in the ultraviolet range above 220 m $\mu$ . When attached to the purine or pyrimidine nucleus, however, they influence the position and intensity of the absorption spectra of the bases. Very little work has been done on the infrared absorption of these compounds (31).

The studies of optical properties of nucleic acid derivatives may serve the following purposes: (a) Compilation of physical constants as an aid to identification and characterization of compounds. (b) Investigation of structural problems. (c) Studies of the location and concentration of polynucleotides in cells *in situ* by means of ultraviolet microspectroscopy. (d) Tracing enzymic reactions attended with a change in the absorption spectrum.

##### A. CHARACTERIZATION OF PURINES, PYRIMIDINES, AND THEIR DERIVATIVES BY ULTRAVIOLET ABSORPTION SPECTRA

Prior to 1935, the ultraviolet absorption of nucleic acid constituents has been studied sporadically by a number of investigators (17,85), but the significance of such measurements was not generally appreciated. Thus, in Levene and Bass' monograph (241) no reference to this subject is found. In the meantime, however, ultraviolet spectrophotometry has become a very important tool in this field.

The structure and position of the absorption spectra were found to be closely dependent upon the pH of the solution. With an in-

crease in acidity a shift toward the shorter wavelengths occurs and in alkaline medium the opposite is observed (see Figs. 2, 3, and 4).

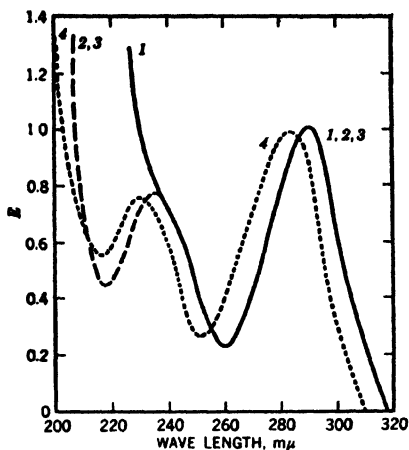


Fig. 2. Absorption spectrum of uric acid (368). Concentration 1.7 milligram per cent: Curve 1, pH 9.1; 2, pH 7.8; 3, pH 6.8; 4, pH 2.0.

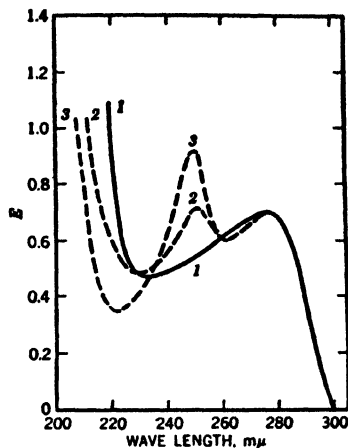
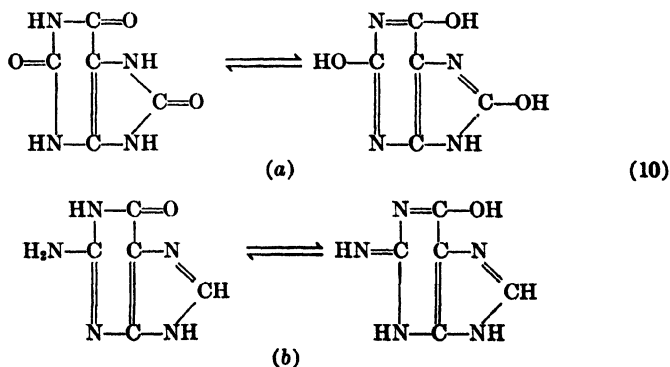


Fig. 3. Absorption spectrum of guanine (179). Concentration 2.2 milligram per cent: Curve 1, pH 9.0; 2, pH 6.8; 3, pH 2.4.



In some instances a change in pH may cause a complete alteration of the spectrum. For example, uric acid shows two absorption bands in acid solution and only one in alkaline solution (368) (see Fig. 2)

TABLE II  
ULTRAVIOLET ABSORPTION OF NUCLEIC ACIDS AND RELATED COMPOUNDS

Compound	Wave-length, m $\mu$	$E_{1\%} \times 10^{-2}$	Solvent or pH	References
Adenine	260	10.2 to 13.4	7	(143,166,264)
Adenosine	260	15.1	7	(143,386)
Adenosine-5'-monophosphate	260	15.8	7	(202)
Adenosine triphosphate	260	16.0	7	(202)
Adenine thiomethylriboside	260	15.5 to 16.9	7	(118,166,333)
Adenine desoxyriboside	260	14.5 to 15.0	7	(151,338)
Diadenosine tetraphosphate	260	24.8	H <sub>2</sub> O	(212)
Guanine	250	9.0	6.6	(165)
Guanosine	250	8.0	7.2	(201)
Guanine desoxyriboside	250	13.0	H <sub>2</sub> O	(152)
Guanylic acid	250	8.2	7	(338)
Hypoxanthine	250	10.5	7.2	(201)
Inosine	250	11.8	7.2	(201)
Inosinic acid	247	11.5	7.2	(202)
Hypoxanthine thiomethyl- riboside	248	11.1	7.0	(333)
Xanthine	270	11.0	H <sub>2</sub> O	(384)
Xanthosine	277	9.0	7	(333)
Xanthosine	265	(5.0)	7	(333)
Xanthosine	248	9.9	7	(333)
Isoguanine	285	17.5	H <sub>2</sub> O	(383)
Isoguanine	278	(15.0)	H <sub>2</sub> O	(383)
Isoguanine	274	16.8	H <sub>2</sub> O	(383)
Isoguanine	252	(6.0)	H <sub>2</sub> O	(383)
Isoguanine	242	10.5	H <sub>2</sub> O	(383)
Isoguanosine	293	14.8	7.0	(333,121)
Isoguanosine	266	(4.7)	7.0	(333,121)
Isoguanosine	247	12.7	7.0	(333,121)
Uric acid	290	12.2	7.2	(201,386)
Uric acid	260	(3.2)	7.2	(201,386)
Uric acid	236	9.0	7.2	(201,386)
Uric acid	221	(6.6)	7.2	(201,386)
Uric acid riboside	290	8.7	H <sub>2</sub> O	(119)
Uric acid riboside	257	(4.0)	H <sub>2</sub> O	(119)
Uric acid riboside	238	8.0	H <sub>2</sub> O	(119)

TABLE II (concluded)

Uracil	261	9.2	H <sub>2</sub> O	(165,166)
Uridine	262	9.8	7.0	(317a)
Uridylic acid	262	10.0	7.0	(317a)
Cytosine	265	6.2	H <sub>2</sub> O	(166,386)
Cytidine	270	8.8	7.0	(317a)
Cytidylic acid	270	9.0	7.0	(317a)
Thymine	261	8.6	H <sub>2</sub> O	(166,385)
Thymine desoxyriboside	265	8.6	H <sub>2</sub> O	(385)
Orotic acid	280	7.0	H <sub>2</sub> O	(293)
Ribonucleic acid (yeast)	260	33.5	H <sub>2</sub> O	(385,417)
Desoxyribonucleic acid (thymus)	260	34.5	H <sub>2</sub> O	(385)

\* Maxima. Minima are given in parentheses. Absorption minima adjoining region of unspecific absorption below 220 m $\mu$  are not listed.

and guanine (179) behaves in a similar fashion (see Fig. 3). This change is ascribed to the existence of the tautomeric forms (uric acid, *a*, and guanine, *b*) given in formulas (10). It is obvious that the pH of the solution must always be controlled when characterization of the bases is attempted. In Table II an attempt is made to compile the absorption maxima of purines, pyrimidines, and their derivatives. Preference has been given to the data obtained with solutions of biological pH. This renders the information valuable with respect to the enzymic methods outlined in Section IV. D. Further data on the relative absorption values of nucleosides and bases have been published recently by Hotchkiss (182). There are different ways of recording absorption spectra. A convenient key for recalculation and comparison of various notations is found in a table compiled by Hogness and Potter (178).

It is regrettable that the spectrophotometric data given by most authors are not accompanied by elementary analyses or other criteria of purity of the compounds. Variation in the content of water of crystallization, or mistaking the salts for free bases may in some instances account for discrepancy of results. The instruments used should also be listed to facilitate judging the accuracy of findings (115).

The extinction of the nucleic acids at 260  $m\mu$  remains somewhat below the figure estimated by adding the individual extinction values of the bases (see Table II). This has been ascribed to the weighting effect of the carbohydrate phosphoric acid substituents and to the particular combination of the units in the polynucleotides (165).

One of the problems encountered in recent work is the existence of nucleic acids in which the four bases do not occur in equimolar concentrations (see Sect. VII. H.). It is unfortunate in this respect that the absorption maxima of guanine, adenine, cytosine, uracil, and thymine are situated so close together. The application of spectrophotometry to the study of aberrations in the composition of some polynucleotides does not seem hopeless, however. Measurements before and after hydrolysis of the bases as well as shifting the pH value would seem necessary. Particularly, deamination would separate the maxima of the purine bases. The validity of conclusions could be checked by an analogous procedure with artificial mixtures of the bases.

#### B. ALLOCATION OF THE CARBOHYDRATE IN PURINE NUCLEOSIDES BY SPECTROPHOTOMETRIC STUDIES

The work of Levene and associates seemed to indicate that the carbohydrate in the purine nucleosides is attached in position 7 of the base (241). Gulland and Macrae reinvestigated the problem,

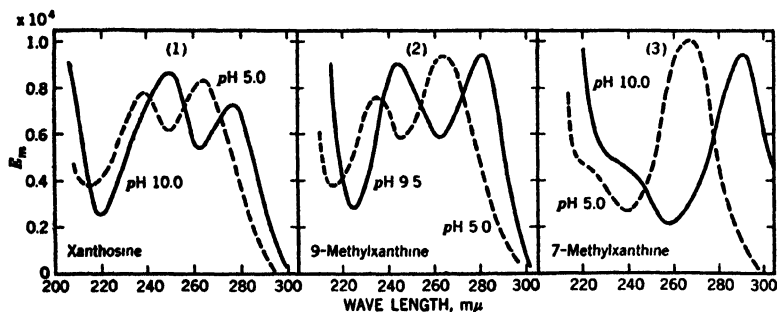


Fig. 4. Absorption spectra of xanthosine and monomethylxanthines (144).

but they were unable to arrive at a decision by methylation experiments (149). They found, however, that purine derivatives methylated in positions 7 or 9 show marked difference in their absorption spectra (144) as can be seen from Figure 4. Comparison of the absorption spectra of the nucleosides with those of their purine constituents methylated in position 7 or 9 showed in all instances close

agreement between the nucleosides and 9-methylpurines, as illustrated in the figure. Besides xanthosine, the following nucleosides were established in this way as 9-purine derivatives by Gulland and co-workers: adenosine and mosine (143), adenine thiomethylpentoside (118), adenine desoxyribose (151), guanosine and guanine desoxyribose (152), uric acid riboside (119), and isoguanine riboside (crotonoside) (121).

### C. APPLICATION OF SPECTROPHOTOMETRY TO PROBLEMS OF QUANTITATIVE CYTOCHEMISTRY

The selective absorption of nucleic acids at  $260\text{ m}\mu$  is so great that it exceeds the absorption of any other cell constituent present in similar or even higher concentration. Thus, on an ultraviolet micro-

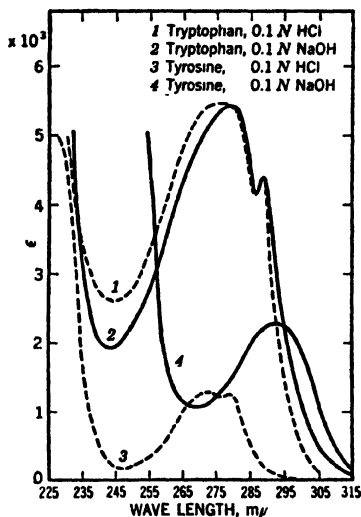


Fig. 5. Absorption spectra of tryptophan and tyrosine (180).

photograph of a tissue section taken with light of wavelength  $260\text{ m}\mu$  the nucleic-acid-rich particles appear very dark against a lighter background. Using this principle, Caspersson has developed, in a monumental series of investigations, microphotographic and spectrophotometric methods which abound with technical finesse and applicability. Only a brief review of this work will be given, since the



results and advances made by Caspersson and colleagues have been reported in several review articles (59-61).

Of the cellular constituents present in concentrations approaching those of nucleic acids only a few proteins show significant specific absorption in the ultraviolet range. This is due to amino acids which contain a resonating substituent, R, in the molecule  $RCH(NH_2)COOH$ , like tyrosine, tryptophan (180), and, to a lesser extent, phenylalanine (see fig. 5). It is apparent from Figure 6 that there is

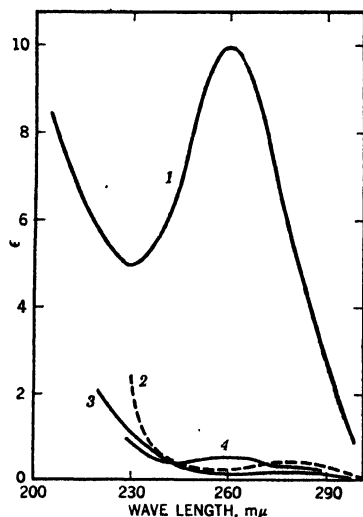


Fig. 6. Absorption spectra of desoxyribonucleic acid and of some proteins from thymus (59): (1) tetrasodium salt of desoxyribonucleic acid, 0.05%; (2) serum albumin, 0.05%; (3) protamine sulfate, 0.5%; (4) histone sulfate, 0.05%.

little interference from these amino acids, since their concentration amounts to only a small percentage in some proteins, and those characteristic of the cell nuclei are particularly low in tyrosine and tryptophan. Moreover, Hammarsten and Caspersson (62) have developed a method of digesting the protein with trypsin *in situ* while the nucleic acid is fixed simultaneously with lanthanum ion. The study of biological material by Caspersson's technique involves the following steps: Observation of the living cell with the ultra-

violet microscope and comparison of the picture with that of the frozen and dried preparation. This is followed by observation and measurements of the same preparation after extraction of interfering substances. Quantitative absorption measurements of objects as small as  $0.6 \mu$  can be made with about 5% accuracy. Below this the light intensities are insufficient for the photographic technique. Therefore, Caspersson has replaced it by a photoelectric method which is more exact. It would go beyond the scope of this review to enumerate the technical details, and the reader is referred to the original papers (58,59).

Unfortunately, the purchase and operation of Caspersson's equipment are beyond reach of the average institute. Since such a wealth of information has been gathered by this technique (61,279), it is not surprising that a simplification of the instruments has been attempted by others (235,319,406). This may be possible at some sacrifice of accuracy, which seems justified in order to provide simple and inexpensive equipment for semiquantitative work to a large number of investigators rather than restrict this most important cytological technique to a very few institutes.

#### D. APPLICATION OF SPECTROPHOTOMETRY TO ENZYME STUDIES

The observation of optical changes attending the reactions nicotinamide  $\rightarrow$  dihydronicotinamide, and isoalloxazine  $\rightarrow$  dihydroisoalloxazine by Warburg and co-workers (415, 418) opened a new era in the study of enzyme reactions which depend on or are linked with these coenzyme nucleotides (287,296,392). The spectrophotometric methods have been described in detail by Warburg (414) and in many instances they have superseded the manometric techniques of enzyme research.

Kalckar has extended this work to reactions in which purines and their derivatives undergo enzymic changes accompanied by alteration of position and height of the absorption maxima (199,200).

The deamination of adenosine derivatives may serve as an example (203). Adenosine triphosphate and adenosine monophosphate show an absorption maximum at  $260 m\mu$  (see Fig. 7). Inosine monophosphate has an absorption maximum at  $250 m\mu$ . At wavelength  $265 m\mu$ , the discrepancy in absorption between adenosine and inosine compounds is particularly large and suitable for measure-

ment. The extinction value of inosinic acid is only 40% of that of the amino compound in equimolar concentration. The opposite holds for wavelength 240  $m\mu$ . Here, inosinic acid absorbs more readily than adenylic acid. Deamination, therefore, results in a decrease of absorption at 265  $m\mu$  and an increase at 240  $m\mu$ , as indicated in Figure 7. The corresponding experiment with adenosine and adenosine deaminase gives the changes represented in Figure 8. Line  $E_{265}$  shows the rate of decrease in density at 265  $m\mu$  which would result

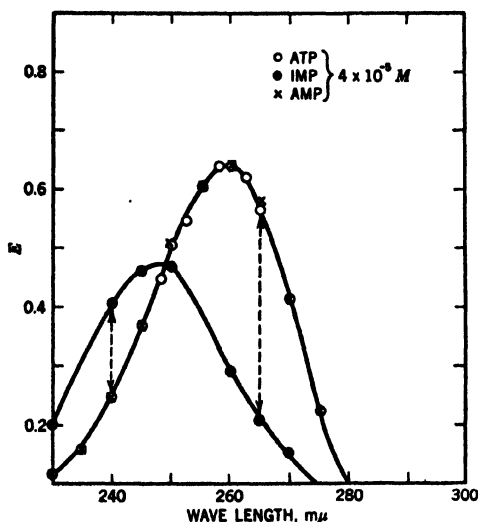


Fig. 7. Ultraviolet absorption of adenosine triphosphate (ATP), inosine monophosphate (IMP), and adenosine monophosphate (AMP) (203).

from conversion of adenosine to inosine, while line  $E_{240}$  indicates the expected increase in density at 240  $m\mu$  due to such a reaction. Disappearance of adenosine and formation of inosine alter the extinction in a linear fashion. It can be seen from the graph that deamination of 10  $\mu g.$  of adenosine per milliliter of solution causes a decrease of the extinction at 265  $m\mu$  by 0.263. Much smaller changes can be measured with the usual instruments and the deamination of less than 1  $\mu g.$  of adenosine can be observed.

Figure 9 illustrates the spectrophotometric basis of studies on

the oxidation of hypoxanthine to xanthine and uric acid (16,202). In addition, the conversion of guanine to xanthine by guanase, and the degradation of uric acid to allantoin by uricase can be observed, since changes of the absorption maxima are involved.

The value of Kalckar's methods rests in the following advantages:

- (a) Very low concentrations of the purine compounds can be studied.
- (b) The reactions can be carried out in the cuvettes and the progress can be measured continually.
- (c) Reactions which themselves are

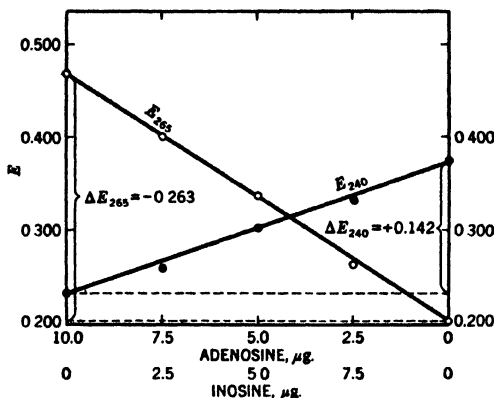


Fig. 8. Optical densities of mixtures of adenosine and inosine at 240 and 265 mμ (203). Concentrations in micrograms per milliliter.

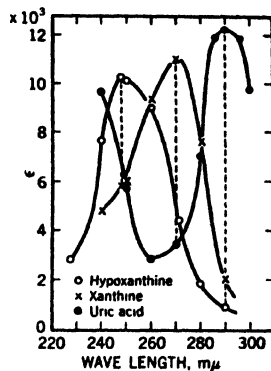


Fig. 9. Molecular extinction curves for some oxy-purines (202).

not attended by a noteworthy optical change may be studied, provided they can be coupled with another step involving such a change. (d) In comparison to older procedures much time is saved.

As an illustration of the last point the study of inosine and guanosine nucleosidase (201) may serve. Formerly, incubation of relatively large amounts of substrate with nucleosidase was necessary. After deproteinizing aliquots at intervals, the purine base had to be removed by a heavy metal precipitation, the excess metal ion had to be removed by hydrogen sulfide, and finally, the carbohydrate in the filtrate as determined by its reducing power gave an indication of the progress of nucleosidase splitting. It is not surprising that these operations caused considerable inaccuracy. More exact results are obtained by Kalckar's technique with the expenditure of a fraction of the time and material. Inosine is incubated with nucleosidase and xanthine oxidase. As soon as hypoxanthine is set free it is oxidized to xanthine and uric acid, which causes the optical changes indicated in Figure 9.

Likewise, the action of nucleosidase on guanosine can be studied by combination of this system with guanase and xanthine oxidase, which results in the sequence:

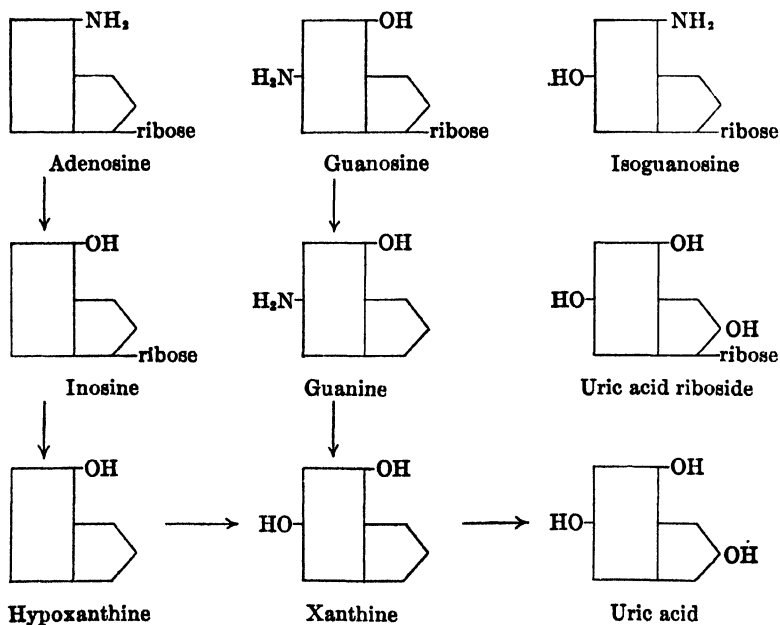


The examples cited above were elaborated with purified enzymes. When crude enzymes or tissue extracts are used, the results are less reliable. This should not present too great difficulty, however, since Kalckar has shown how the interference of tissue constituents may be overcome in some instances (202).

## V. Nucleosides

### A. PURINE RIBOSIDES

The following purine ribosides are known: adenosine, guanosine, inosine (hypoxanthine riboside), xanthosine, uric acid riboside, and isoguanine (hydroxyadenine) riboside (see formulas 11, which



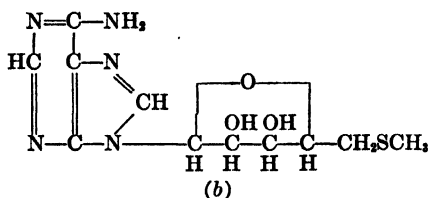
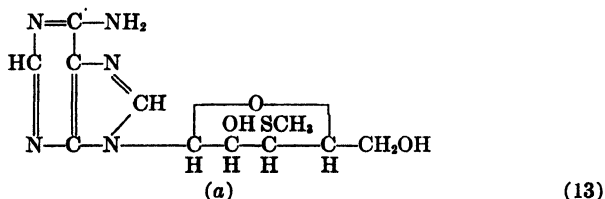
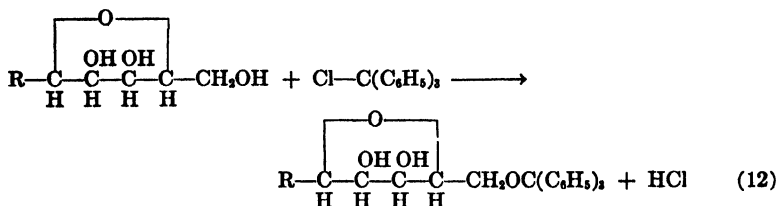
illustrate the purine nucleosides and their catabolism). The biological significance of adenosine and guanosine is apparent from their occurrence in pentose nucleic acids, and the former is also part of several coenzymes. Inosine originates from adenosine by action of the widely distributed adenosine deaminase. Dietary adenosine, for example, is converted prior to absorption by the deaminase which is abundant in the small intestine (34,203). Xanthosine has not as yet been isolated from biological material, but its function as an intermediate in the formation of uric acid riboside and perhaps in the conversion of adenosine to guanosine (see page 463) may be suspected. Uric acid riboside was discovered in blood many years ago by Davis, Newton, and Benedict (82,303), and more recently it has also been found in liver (119), but its biological significance is still obscure. The enzymic degradation of purine nucleosides is generally believed to take its course via the free purines from hypoxanthine or xanthine on, as outlined in formulas (11). There may be an alternative path leading to uric acid riboside, but the enzyme responsible for this has not been found. Xanthine oxidase, which brings about the changes, hypoxanthine  $\rightarrow$  xanthine  $\rightarrow$  uric acid, does not oxidize inosine or xanthosine (96), and uric acid riboside is resistant to uricase (356).

Isoguanosine (crotonoside) has been discovered by Cherbuliez and Bernhard (67) in croton seeds (*Tigllium croton*). This unusual material has attracted the interest of biochemists for several reasons: Besides croton oil it contains the extremely toxic protein crotin (104) and shows highly allergenic properties; hence, the preparation of crotonoside from the seeds requires precautions which are unusual in nucleotide chemistry (373a). The structure given in formulas 11 was suggested by Cherbuliez (67) and corroborated by Spies (374).

In all purine nucleosides discussed here the ribose is attached to position 9 of the purine nucleus (see Sect. IV. C). The linkage is  $\beta$ -glucosidic in the case of adenine (83), and by analogy this may be inferred for the other purine ribosides. A furanoid ring structure has been proved by methylation experiments (36,252) and by reaction with triphenylchloromethane (trityl chloride). The latter reagent yields a triphenylmethane derivative only when a primary alcoholic group is available (see formula 12). A nucleoside with pyranoid structure would not give the triphenylmethane derivative.

Adenosine and guanosine can be converted readily into inosine and xanthosine; hence a furanoid structure may be assumed also for the latter two nucleosides. The ring structure of uric acid riboside and of isoguanosine has not been established.

Among the nucleosides, adenine thiomethylpentoside should be mentioned. So far it has been found only in yeast (241,280,389,391),



but no extensive search for other sources is recorded in the literature. The structure according to Levene and Sobotka (250) is given in formula (13a), while Suzuki, Odake, and Mori (390,391) favor a configuration represented by formula (13b). The arguments of the latter authors appear more satisfactory, and recent experiments by Wendt (419) favor structure b. The biological significance of this compound is still obscure. Lipmann has suggested (258) a role as cotransmethylase, but experiments with tissue preparations and bacteria have so far failed to substantiate this assumption (339,342).

## B. PURINE DESOXYRIBOSIDES

In their monograph, Levine and Bass stated (241): "One might fear that the progress of our knowledge on the desoxypentosides will be slow due to the difficulty experienced in securing material." How true has time proved this statement! The number of investigators who have submitted to the cumbersome task of preparing these compounds has indeed remained very small. Levene and Bass further wrote: "Fortunately, much about the details of their structure can be inferred from our knowledge of the chemistry of the ribonucleosides." In many instances experimentation has substantiated assumptions made in this spirit, but in the present era of positivism in the nucleic acid field some investigators will favor a more cautious attitude.

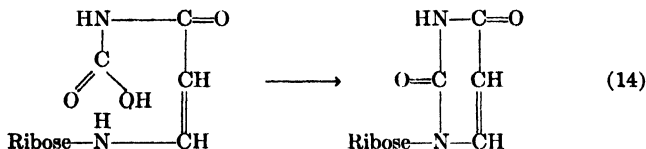
In the purine desoxyribosides the linkage between sugar and base is much more sensitive than in the ribosides, although the place of substitution is nitrogen atom 9 of the purines in both instances. The only feasible procedure of isolation is enzymic hydrolysis. For this a source of strong phosphatase and nuclease action, devoid of nucleosidase action, is required. Mammalian small intestine, for example, fills these specifications, and the original technique of Levene and London (246), which employed dogs with an intestinal fistula, has been superseded by *in vitro* methods which we owe to Thannhauser and his school (27,213,214,218,397). The following compounds were obtained: desoxyadenosine, desoxyguanosine and desoxyinosine. (Simplified terms like desoxyadenosine instead of adenine desoxyriboside have been proposed by Brady (34) and have found wide acceptance.) Desoxyinosine is formed from desoxyadenosine in the course of the enzymic hydrolysis, unless the deaminase present in the enzyme preparation is blocked by addition of heavy metal ions (34). The assignment of furanoid structure and the assumption of  $\alpha$  or  $\beta$  nature of the glucosidic linkage are arbitrary.

## C. PYRIMIDINE RIBOSIDES AND DESOXYRIBOSIDES

The pyrimidine ribosides are cytidine (cytosine-3-riboside) and uridine (uracil-3-riboside). The allocation of the sugar to the position 3 of the base as suggested by Levene and Bass (241) has remained uncontested (44). The furanoid structure of the carbohydrate could be established (183,253) and  $\beta$ -ribosidic linkage is probable (83).

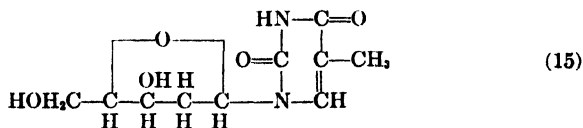


Very interesting results concerning the biosynthesis of the pyrimidine nucleosides have come from studies on *Neurospora* mutants and related organisms (22,134). Some of them utilize the free pyrimidine bases very poorly compared to the nucleosides. This suggested to Loring and Pierce (271) the possibility that the bases are not intermediates in the formation of nucleic acids. Mitchell and co-workers (291,292) have extended the studies and found that oxalacetic acid may contribute to the carbon chain of pyrimidines. There is an indication (291) that in *Neurospora* an open chain aliphatic glycoside of ribose is an intermediate in nucleoside synthesis as shown in formula (14). In some of the deficient strains



orotic acid can be utilized for pyrimidine nucleoside synthesis. It remains to be seen to what extent the findings on *Neurospora* can be duplicated in other organisms.

Pyrimidine desoxyribosides show greater stability of the glucosidic linkage than purine desoxyribosides. Attachment of the sugar in position 3 of the bases cytosine and thymine has been proved by methylation experiments (44), and in thymidine a furanoid



ring structure could be established (255). The identity of the sugar with D-2-desoxyribose remains to be demonstrated. It seems possible that desoxyuridine is formed in nature as a precursor or degradation product of cytosine desoxyriboside, but uridine desoxyriboside has never been found, and no deaminase capable of converting desoxycytidine into desoxyuridine has been isolated.

Thymidine (thymosine, thymine desoxyriboside) (see formula 15) has attracted considerable interest recently. As reported in Section

III. E., it was found to be an essential nutrilit for some organisms. If provided in a concentration of several micrograms per 10 ml. of medium, optimum growth of lactobacilli occurs even in the absence of liver concentrate or vitamin B<sub>12</sub>. Wright, Skeggs, and Huff (427) believe that the defect in the organism may concern the nucleoside phosphorylase (nucleosidase), which brings about combination of thymine and desoxyribose, and vitamin B<sub>12</sub> is supposed to act as coenzyme in this process. Since this vitamin causes a favorable response in cases of Addisonian pernicious anemia (324), Wright suggests (427) that the disorder may be inability to synthesize certain nucleosides from their parent bases. Thus it would appear that the curative effects observed in this disease with folic acid arise from increased thymine synthesis, which by mass action yields more thymidine. The effectiveness of large amounts of thymine in pernicious anemia may be explained similarly.

The correlation of folic acid and vitamin B<sub>12</sub> with the biosynthesis of nucleic acid constituents is one of the most fascinating recent developments in the field. The ever-increasing number of notes on this subject indicates that important advances are imminent (175a, 319a, 364a, 365a).

#### D. DEAMINASES

Amination of the purine or pyrimidine nucleus seems to be one of the last steps in assembling the nucleosides and nucleotides *in vivo*. Conversely, decomposition starts with deamination. Formation of ammonia from nucleic acids upon incubation with tissue enzymes had been observed by early workers (191, 192, 334, 335), but little significance was attributed to this process beyond registration of an autolytic phenomenon. More intense study of the enzymes began with the investigations on adenylic acid deaminase and adenosine deaminase by Schmidt (343). The reason for concentrating on these two enzymes in preference to other deaminases may have been the discovery of adenylic acid by Embden (109, 176, 185) and the role attributed to ammonium ion in muscular contraction at that time. Szent-Györgyi reports in his recent treatise on the *Chemistry of Muscular Contraction* (393) that a linkage exists between the amino group and one of the phosphoric acid groups of adenosine triphosphate. Resolution of this bond is accompanied by deamination.

Adenosine deaminase has recently been obtained by Brady (34)

and by Kalckar (203) in purified state from intestinal mucosa. This source is particularly rich in the enzyme, and one might suspect a function to protect the mammalian organism from the strong pharmacological action of dietary adenosine. The deaminase follows the intestinal phosphatase through most of the purification steps outlined by Schmidt and Thannhauser (332,352) and can

TABLE III  
SPECIFICITY OF PURINE NUCLEOSIDASE AND ADENOSINE DEAMINASE

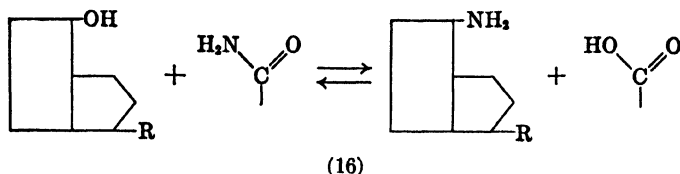
Compound examined	Purine nucleosidase splitting <sup>a</sup>	Adenosine deaminase splitting <sup>a</sup>
6-Aminopurine riboside (adenosine)	—(96,201)	+(34,191,203,343)
2-Hydroxy-6-aminopurine riboside (isoguanosine, crotonoside)	—(333)	—(333)
2-Amino-6-hydroxypurine riboside (guanosine)	+(96,201)	—(343,345)
2-Amino-6-oxypurine desoxyriboside (desoxyguanosine)	+(215)	—(343)
6-Aminopurine desoxyriboside (desoxyadenosine)	—(338)	+(34)
6-Aminopurine thiomethylriboside	—(333)	—(116,333)
6-Aminopurine-5'-phosphoribose (adenylic acid from coenzymes)	—(343)	—(343)
6-Aminopurine-3'-phosphoriboside (adenylic acid from ribonucleic acids)	—(343)	—(343)
6-Aminopurine (adenine)		—(191,203)
6-Hydroxypurine desoxyriboside (desoxyinosine)	+(215)	
6-Hydroxypurine riboside (inosine)	+(96,201)	
2,6-Dihydroxypurine riboside (xanthosine)	—(96,201)	
2,6,8-Trihydroxypurine riboside (uric acid riboside)	—(201)	

<sup>a</sup> Reference numbers are given in parentheses.

be separated eventually by fractional adsorption on alumina C-gamma gel. It is an enzyme of rather high specificity. The influence of modifications of the adenosine molecule is apparent from the data given in Table III. Interference with the action of the enzyme is due to modifications in the purine nucleus as well as in the carbohydrate moiety (333). Adenine itself is not deaminated by this enzyme; it does not interfere with adenosine deamination by competitive inhibition (380). Attachment of ribose or desoxyribose

in position 9 of the purine is required, but no substituent in the carbohydrate is compatible with deamination. The chemical composition of adenosine deaminase should be studied further (273, 332,388). Zittle reports the absence of carbohydrate in the molecule (433), while intestinal phosphatase (352) appears to be protected by carbohydrate units in its structure against attack from proteolytic enzymes of the intestinal tract.

The reversibility of the adenosine and adenylic acid deamination is a process which has been subject to speculation for a long time (9,219,225). Since no reaction of inosine with an excess of ammonium ion in the presence of the enzyme could be observed, it became clear that a special donor of amino groups must participate, and quite early, glutamine or asparagine were assumed to be involved (205,373) in a fashion indicated by formula (16), which illustrates



amination of hypoxanthine derivatives by glutamine or asparagine ( $R = \text{ribose or ribose-5-phosphate}$ ). Recently, some progress in verifying this scheme has been reported by Elliott (106). He obtained an enzyme preparation from sheep brain which catalyzed the reaction  $\text{L-glutamic acid} + \text{adenosine triphosphate} \rightleftharpoons \text{L-glutamine} + \text{inosine diphosphate} + \text{phosphoric acid}$ . Magnesium ion was necessary in this process, and the amount of phosphate liberated corresponded to the transfer of amino groups. In the presence of hydroxylamine, a hydroxamic acid,  $\text{HOOCCH}(\text{NH}_2)\text{CH}_2\text{CH}_2\text{CONHOH}$ , was formed which proved particularly suitable for analytical observation of the process. The findings were extended in collaboration with Gale (107) to *Staphylococcus aureus*.

The interconversion of glutamine, inosine phosphate, glutamic acid, and adenosine triphosphate may be an example of a more general type of reaction by which hydroxypurine derivatives are aminated, but much remains to be studied in this field.

Adenosine-5'-phosphate deaminase shows high specificity in that it does not act on adenosine polyphosphates, cozymase, adenosine-3'-

phosphate, or desoxyadenylic acid (216). Its action is retarded by inorganic phosphate and by some specific inhibitors like phenol or barbital (74,272,380,388).

Of interest are the strong pharmacological effects of adenylic acid and its derivatives in mammals as described first by Drury and Szent-Györgyi (98). In general, these substances produce cardiac slowing, depression of the nodes and conduction of the heart, and lowering of the blood pressure, largely through direct peripheral vasodilatation; inhibition of intestinal muscle, and stimulation of uterine muscle (24,97,111,116,274,315) have also been found. The effects are observed after injection of amounts which are incomparably smaller than the biological concentration of the adenosine compounds in the organism, and one wonders by what mechanisms the latter are neutralized under normal conditions. The pharmacological phenomena have been correlated with deamination (315). In accordance with this, some explanation would come from the fact that adenosine and its homologues occur in the organism predominantly in a form not subject to deamination. Some of it is built into polynucleotides, some occurs as adenosine polyphosphate, or in stable combination with protein (221) as for example in muscle phosphorylase, where the linkage between the protein and adenylic acid prevents deaminase action (76). Isoguanosine and adenine thiomethylpentoside are not deaminated by adenosine deaminase *in vitro*, but they show pharmacological activity. This seems to be the only indication, so far, of their interconversion with adenosine (116,117,315).

Pharmacological activity sets a limit to the therapeutic use of adenosine compounds, which has been suggested in some states of deranged metabolism involving the adenine nucleotides (376). On the other hand, advantage has been taken of the pharmacological properties to relieve certain spastic conditions (162) and reference should be made to the role of adenosine derivatives in shock (204), as intercellular growth-promoting (wound-healing) hormone (262,263), and in a variety of other pathological conditions (162,208,404).

Compared to the adenylic acid and adenosine deaminases little attention has been given to other purine and pyrimidine deaminases. They have been isolated in crude state only, and, no doubt, some revisions will come from extended studies. Deamination of adenine by adenase is a process restricted to lower organisms (99,100,132, 380) with few exceptions (30,295). Guanase (174) has been studied recently by Kalckar (202) (see Sect. IV. D.). The deamination of guanosine and guanylic acid has been investigated by Schmidt (344,345), who suspects that the liberation of ammonia must be attributed to combined action of nucleotidase, nucleosidase, and guanase. Cytidine deaminase (154,155) is interesting because it does not act on desoxycytidine, surpassing in this respect even adenosine deaminase in its specificity. No cytidylic acid deaminase

has been described yet, and the mammalian organism cannot deaminate cytosine (64a,216).

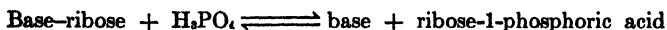
Compared to the ribosides our knowledge on the deamination of desoxyribose nucleosides and nucleotides is lagging. Deamination of desoxyribonucleic acid was observed by Thannhauser (397), but it is probable that disintegration to smaller units precedes deamination (136). Studies on ammonia formation and phosphate mineralization of nucleic acids with crude enzyme mixtures involve many variables, which make results rather uncertain. For example, the ammonia and phosphate may escape detection by being utilized in other processes and, furthermore, erroneous conclusions may come from the fact that other speed-limiting reactions may be prerequisite to ammonia and phosphate liberation. It seems hardly justified, therefore, to use nucleic acids as substrate in comparing the deaminating and dephosphorylating power of various tissues.

#### E. NUCLEOSIDASES (NUCLEOSIDE PHOSPHORYLASES)

The old concept which assumed the existence of only one nucleosidase for all ribosides proved untenable. The erroneous conclusions may have been due to the use of crude enzyme preparations and of unreliable analytical procedures. A noteworthy investigation of Dixon and Lemberg (96) has clearly demonstrated the restricted range of "purine nucleosidase," and the use of this term should be understood now to imply action toward guanosine, desoxyguanosine, inosine, and desoxyinosine only (see Table III). Other purine nucleosides are not split by this enzyme (201,333), and it is probable that in higher animals the metabolism of the nucleosides always proceeds via inosine and guanosine as indicated in formulas (11). A similar scheme of degradation of the desoxyribose nucleosides is probable, but rigorous proof is still lacking. It has been shown by Deutsch that pyrimidine nucleosidase is a distinct entity attacking only uridine, cytidine, and thymidine (84,215).

Much progress in the purification of purine nucleosidase and in analytical methods has been made by Klein (215). The most significant of his observations is that phosphate ion is required for nucleosidase action, but no explanation for this was offered. The occurrence of a phosphoroclastic split was suggested by Lipmann (259), but it remained for Kalckar to provide the experimental

evidence. The splitting of guanosine and inosine, according to Kalckar (201), may be described by the equation:



The state of the equilibrium depends on the phosphate concentration; an increase furthers splitting. The preparation and some properties of ribose-1-phosphate have been described briefly in Section III. H. Its isolation has made possible the first successful enzymic synthesis of a nucleoside (inosine) *in vitro* according to the equation given above (201). Unfortunately, nucleosides cannot be made on a large scale by this method, since ribose-1-phosphate is an extremely rare and labile compound. For the synthesis and degradation of pyrimidine and deoxyribose nucleosides an analogous role of phosphate is indicated by the experiments of Klein (215) and confirmation of this has come recently (281).

#### F. CHEMICAL SYNTHESIS OF NUCLEOSIDES

Experiments on chemical synthesis of nucleosides appear particularly important, because they are the first step in providing the structural units from which nucleic acids may perhaps be synthesized in future. The phosphorylation of nucleosides to nucleotides has been accomplished repeatedly, and the techniques employed range from merely exposing a nucleoside to the uncontrolled action of a phosphorylating agent to elaborate experiments with shielding the OH groups of the sugar in unconcerned positions (12a,14a). It is an unfortunate habit of some investigators to refer to these phosphorylations of natural nucleosides as syntheses of nucleotides. One should remember that the synthesis of the organic part of the molecule is incomparably more important and difficult.

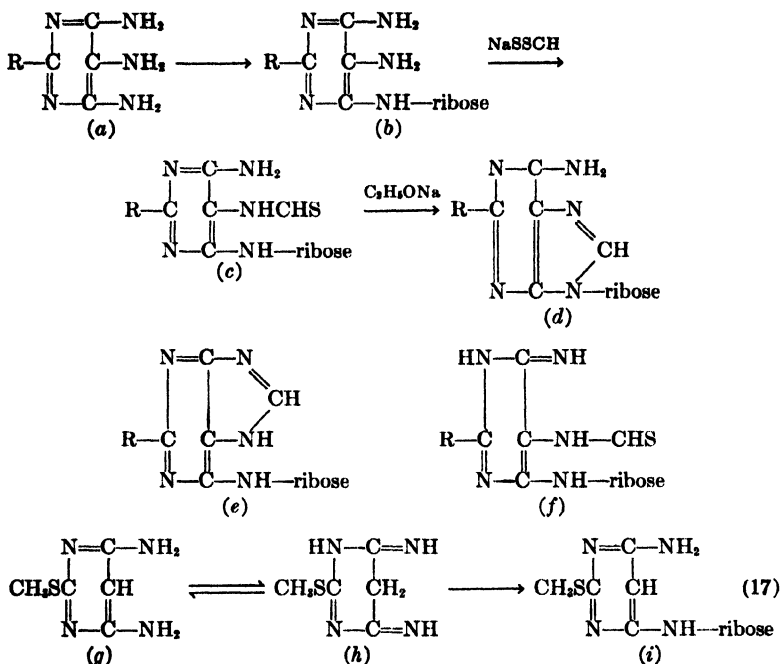
The first attempts to unite acetobromo sugars with purines and pyrimidines are reviewed by Levene and Bass (241). This work has been extended by valuable contributions of Hilbert and co-workers (168-170,410). The recent work of Todd, Lythgoe, and associates seems to open a new era in this field. These authors concentrated on the synthesis of adenosine, a very cogent choice, since the identity of the synthetic and natural compounds may be checked by a number of specific biological tests in addition to the chemical identification. Phosphorylation of the synthetic nucleoside to adenylic acid and study of its coenzyme function, or the specific

pharmacological action of the product can be employed to establish the identity, while comparable biological tests for the other purine nucleosides are not known. The following description can give only the gist of the admirable work of Todd, Lythgoe, and co-workers, who have utilized present day organic chemistry to its very limits. The senior investigators of the group have given a more detailed account of the work in two reviews (275,402).

In early synthetic experiments leading to simple nonbiological nucleosidelike compounds, the linking with the carbohydrate was accomplished by using the acetohalogeno sugar (241). This method gave a number of model compounds in which the place of the glycosidic linkage often remained ambiguous. It could not be used in attempts to synthesize adenosine, since positions 6, 7, and 9 of adenine are alternatives of attachment. Besides, acetobromoribose is an almost inaccessible compound. The Cambridge investigators, therefore, designed an entirely new route which is illustrated briefly and in a somewhat simplified form in formulas (17). The sugar is introduced into the aminopyrimidine, *a*, which is an intermediate in Traube's purine synthesis (403). The imidazole ring is closed subsequently, and dithioformate is used instead of the conventional formic acid. This variation permits ring closure, *b,c,d*, under mild conditions so as to leave the sugar-base linkage unimpaired.

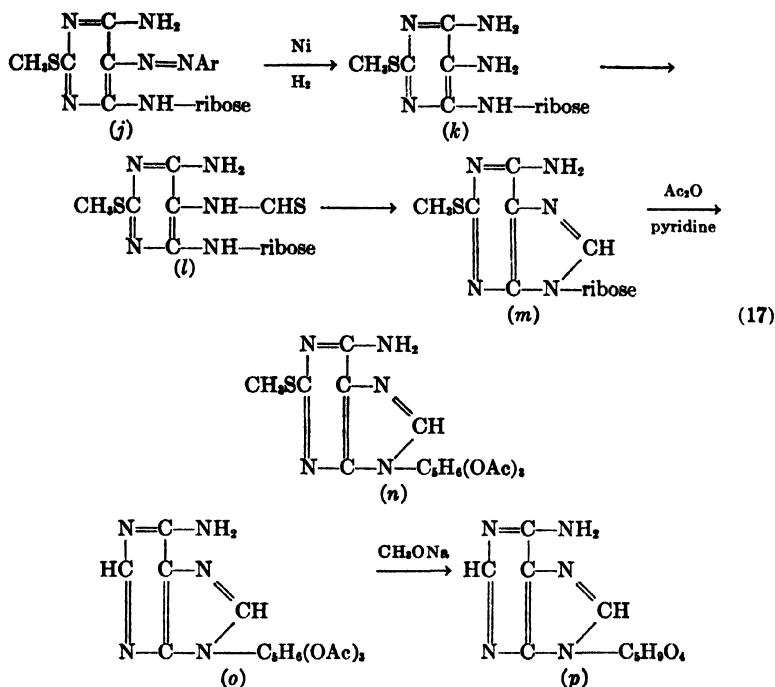
The obstacles encountered in this apparently simple scheme were numerous. In the first place, the ring may close to yield product *e* instead of *d*. Fortunately, model experiments with simple substituents instead of ribose showed that compound *c* occurs predominantly in the iminodihydropyrimidine form, *f*, which favors the course of reaction given by *c* and *d*. The preparation of the 5,6-diamino-4-glycosidaminopyrimidine proved difficult. Preliminary experiments showed the necessity of introducing the sugar into the 4,6-diaminopyrimidine, *g*, with subsequent attachment of the amino group in position 5. Compound *g* behaved essentially like the tautomeric iminodihydropyrimidine, *h*, but presence of a suitable substituent, R, was found to render at least one of the basic groups in position 4 or 6 reactive in the amino form. A thiomethyl group at carbon atom 2 was found particularly advantageous. Condensation of compound *g* with ribose proved possible in boiling water-free ethyl alcohol in the presence of acid catalysts. The glycoside *i* is very sensitive. Introduction of an amino group in position 5 of





the pyrimidine nucleus could be brought about in some instances by nitrous acid yielding a reducible 5-nitroso derivative, or better, by using a reactive diazonium compound like 2,5-dichloroaniline diazotate, which couples at position 5 as formulated in *j*. Catalytic reduction gave the desired amino group, *k*, and cyclization was accomplished with sodium thioformate, *l,m*. The thiomethyl group could be removed from position 2 by Raney nickel catalyst according to Mozingo and co-workers (297) as shown in formulas (17) (*n,o,p*).

Examination of the adenine riboside, *p*, showed that it was an isomer of adenosine, containing a pyranoid instead of a furanoid ring. Todd and colleagues have begun further experiments to overcome this difficulty. Their endeavor has been rewarded by success recently (83a). A somewhat different route was used. Condensation of the silver salt of 2,8-dichloroadenine with acetochloro-D-ribofuranose, followed by deacetylation gave 2,8-dichloro-9-β-D-ribofuranosido-adenine. On hydrogenation with a palladized barium sulfate catalyst



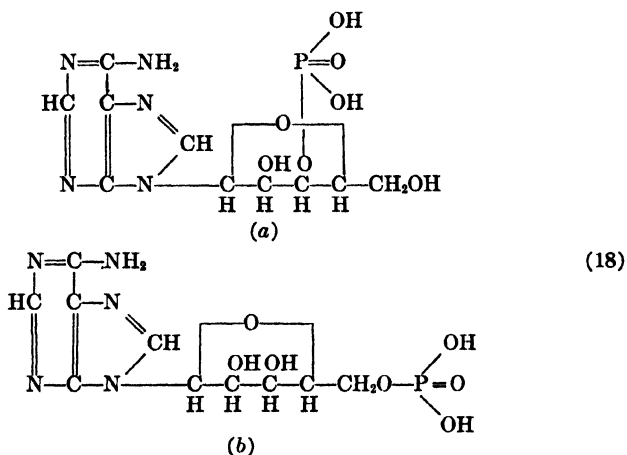
this compound yielded 9- $\beta$ -D-ribofuranosidoadenine which was identical with natural adenosine. Corresponding work in the pyrimidine nucleoside field seems to be somewhat simpler. A successful synthesis of cytidine has been announced by Lythgoe and Todd (183,275). The difficulty in this work was to secure acetobromoribofuranose, but it was overcome by improving the methods of Brederick (43).

## VI. Nucleotides

### A. RIBOSE NUCLEOTIDES

The main structural difference between coenzyme nucleotides and the units of ribonucleic acid seems to be the position of the phosphoric acid in the molecules. In the coenzymes position 5 of the carbohydrate is favored by nature, while the hydrolytic fragments

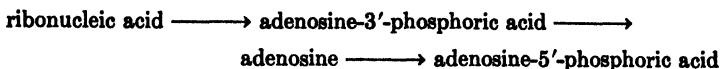
of ribonucleic acid are 3'-phosphate esters (see formulas 18). Attachment of the phosphoric acid at carbon atom 3 of the ribose has been proved for the adenine and guanine nucleotides (241), and the same position of the phosphoric acid in the pyrimidine ribose nucleotides is ascertained by synthesis (254).



In formulas (18) are shown: (a) adenosine-3'-phosphoric acid, a constituent of ribose polynucleotides. Other names are yeast adenylic acid, h-adenylic acid, and syn-adenylic acid. (b) adenosine-5'-phosphoric acid, a structural unit of adenosine triphosphate, adenine nicotinamide dinucleotides and adenine riboflavin dinucleotide. Other names are muscle adenylic acid, t-adenylic acid, erg-adenylic acid, myo-adenylic acid, and AMP.

The borderline between coenzymes and the other ribonucleotides is not altogether sharp. There are some bridges which seem to link these two groups. For example, the interconversion of the two types of adenylic acid has been suggested by Ostern and co-workers (312-314). They found that adenosine is readily phosphorylated to adenosine-5'-phosphate by yeast in the presence of a suitable phosphate donor. Since yeast contains the enzymes necessary for splitting and dephosphorylating the adenylic acid units from ribonucleic acid, special significance was attributed to the latter compound as a storehouse dispensing the units for coenzyme synthesis. It seems, however, that not all types of yeast (338) phosphorylate

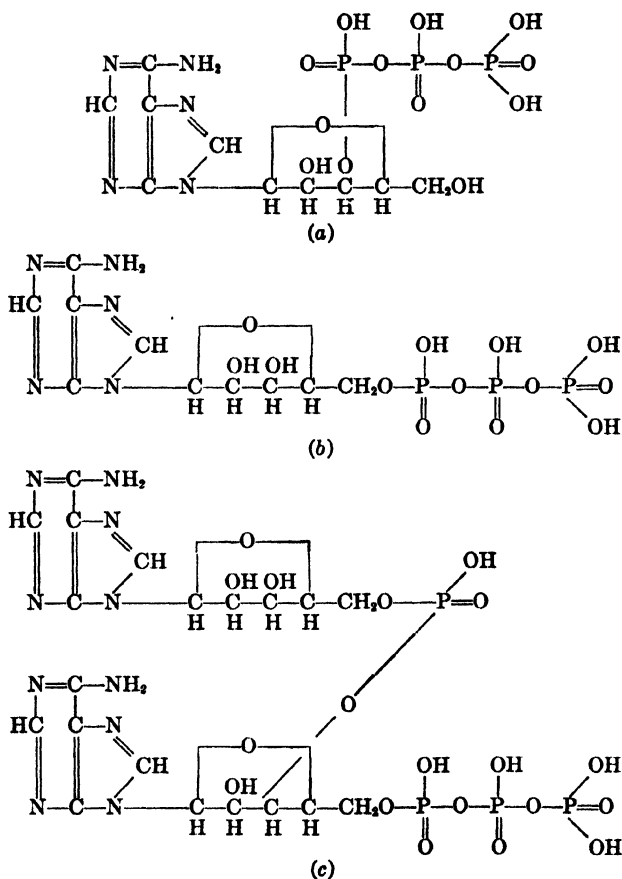
adenosine so rapidly and in such amounts as found by Ostern, and it remains to be seen, therefore, whether the reaction:



has importance beyond being a phase of the dynamic state of the nucleic acid constituents in yeast. Experiments with labeled adenine have shown that in the rat no conversion of adenylic acid from polynucleotides into adenosine triphosphate takes place (51,205).

More recent observations seemed to provide another link between coenzymes and ribonucleic acid, the latter again playing a role as potential coenzyme source. The liberation of guanine from the molecule by a special nucleophosphorylase was assumed, and coenzyme activity of the guanine was reported (72). The interpretation of these results, however, has been modified recently by Colowick (71). Of particular interest are reports by LePage, Umbreit, and Albaum (4-6,237,407), indicating the existence of an isomer of adenosine triphosphate in some autotrophic microorganisms. The structure assumed for this compound (*a*) is contrasted with the conventional adenosine triphosphate (*b*) in formulas (19). The new compound has been isolated from sulfur bacteria (*Thiobacterium thiooxidans*) and from plant material. The evidence for a structure as given in formula (19) is still very limited. It is based on the following observations. The compound contains readily hydrolyzable phosphoric acid like conventional adenosine triphosphate, but the "acid resistant" phosphoric acid group linked to the sugar shows some difference in rate of mineralization on prolonged heating compared to the corresponding phosphate in adenosine-5'-triphosphate, and the response to the orcinol reagent in the pentose determination differs (7). The composition is indicated by demonstration of adenine, pentose, and phosphoric acid in a ratio 1/1/3, but no elementary analyses or titration curves have been reported. Also, that the compound is a homologue of adenosine-3'-monophosphate is assumed merely from the speed of hydrolysis and its nonidentity with the 5'-substituted compound. Indeed, the discovery of a hybrid adenosine polyphosphate in yeast by Kiessling and Meyerhof (212) containing presumably a carbohydrate-3-phosphate ester linkage (see formula 19c) seems to the reviewer a suggestive argument for the existence of Umbreit's adenosine-3'-

triphosphate. It will be interesting to see whether the enzymes of thiobacteria can proceed with transphosphorylations when the readily available adenosine-3'-monophosphate is supplied as co-enzyme. The problem is of rather recent date and the source material

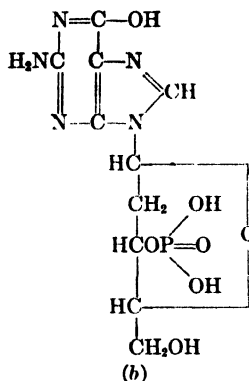
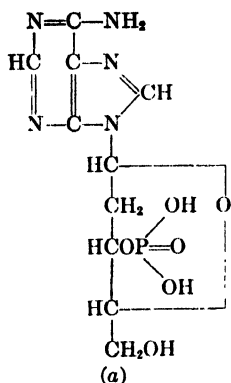


for isolation of the coenzyme is not readily available in quantity. Umbreit suspects that the new compound occurs only in chemo- and photosynthetic organisms, whose sole source of carbon can be carbon dioxide (407). The contention that adenosine-3'-triphosphate is an evolutionary primitive form of the coenzyme is very attractive.

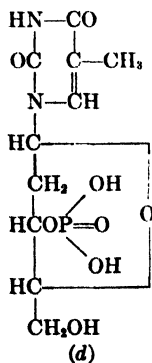
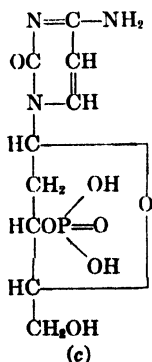
An open mind should be kept toward the acceptance of the existence of the compound; its structure will have to be proved by chemical experiments as meticulous as those of Lohmann (260) with respect to the "old" adenosine triphosphate. The problem is so important that no effort should be spared toward a satisfactory solution.

### B. DESOXYRIBOSE NUCLEOTIDES

The location of the phosphoric acid in the desoxyribose nucleotides is an unsolved problem. By acid hydrolysis of the polynucleotide from thymus gland thymine desoxyribose diphosphate and diphosphodesoxycytidine could be isolated (239,243,398). The method of preparation precludes a pyrophosphate bond, but it could be shown that both phosphate molecules are linked to the sugar (239). The uniformity of these split products has been questioned by Brederick



(20)



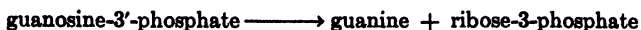
(40). In view of the fact that our knowledge on the desoxyribose nucleotides is scanty in some respects, great restraint should be observed in considering formulas (20) as more than tentative structures in which much is inferred from observations made on ribonucleotides. Attempts at a detailed formulation of desoxyribose polynucleotides seem impetuous under these circumstances. The formulas shown in (20) are: (a) desoxyadenylic, (b) desoxyguanylic, (c) desoxycytidylic, and (d) desoxythymosinic acid.

The obstacle to progress in the investigation of these compounds is the unusual sensitivity of the sugar. One may wonder which methods will succeed eventually to solve the problems in this field. Important strides are being made presently in the chemical study of desoxy sugars (186), and much hope rests in the isolation of individual enzymes concerned with the biosynthesis of desoxy nucleotides. Studies on their specificity using synthetic model compounds may reveal important clues. Biologists have suggested an interconversion of ribo- and desoxyribonucleic acids. If the change should be found to occur with the sugar *in situ* at or above the nucleotide stage, much information gained on ribose nucleotides could be transposed with justification to desoxyribose nucleotides, and the consequences would be far reaching. Unfortunately, it seems more likely that complete breakdown into the bases, sugar, and phosphate units occurs with subsequent reassembling to a structure containing the other sugar and the modification of one of the pyrimidine bases.

### C. NUCLEOTIDE-N-RIBOSIDASE

Early work on the enzymic degradation of nucleotides indicated that phosphatase action always precedes enzymic dissolution of the glycosidic bond (241). In accord with this concept is the failure of purified nucleosidase preparations to split guanylic acid or inosinic acid. In 1936, a series of papers by Ishikawa and Komita began to appear (184,223), which seemed to necessitate a revision. It was observed by these authors that nucleotides upon prolonged incubation with crude tissue enzymes are split at the glycosidic linkage without an increase of the inorganic phosphate in the medium. The Japanese investigators assumed, therefore, the exist-

ence of an enzyme, nucleotide-*N*-ribosidase, capable of splitting guanylic acid, for example, according to the equation:



The carbohydrate phosphoric acid ester isolated from the reaction mixture was not very well characterized; in particular, no data on its optical rotation and its hydrolysis by acid were given. The position of the phosphoric acid, therefore, remained open to question. In the light of recent developments another interpretation of the results of Komita appears possible. According to this, the nucleotide is dephosphorylated, the resulting nucleoside undergoes cleavage by nucleoside phosphorylase, and the resulting ribose-1-phosphate is stabilized to ribose-5-phosphate as discussed in Section III. I. This scheme (413) would fit the analytical data of Komita, obviating the necessity of assuming a nucleotide-*N*-ribosidase. The existence of the latter is not disproved, however, and it will have to be accepted as soon as it is possible to isolate ribose-3-phosphate from the reaction mixture.

## VII. Polynucleotides (Nucleic Acids)

A remarkable recent development in the nucleic acid field is the tendency of many investigators to question the existence of distinct tetranucleotides as structural units of polynucleotides, and even their appearance in the course of enzymic or chemical decomposition of the nucleic acid molecules is contested. Consequently, the search for the order in which the bases are linked in the hypothetical tetranucleotide has lost some of its importance. Moreover, it is believed now by many investigators that the bases in nucleic acids do not occur in equimolar proportion (65).

It is of interest to review in retrospect the evidence which had been brought forth in motivating the tetranucleotide hypothesis. Sensitized by the critical attitude now in vogue, the reader of the early literature is amazed at the unwarranted acceptance of some theories and generalizations in the past. It is most encouraging that the pendulum has swung to the opposite direction, but, so far, more has been accomplished in undoing the older concepts than in accumulating data which would provide a satisfactory new formulation. An explanation of the tetranucleotide hypothesis may be that a majority of the early workers agreed that the



TABLE IV  
MOLECULAR WEIGHTS OF SOME NUCLEIC ACID PREPARATIONS

Material examined	Method	Mol. wt. found	Investigator and ref.
Desoxyribonucleic acid, thymus gland (cattle). Prepared in neutral soln., low temp.	Streaming birefringence, ultracentrifuge	500,000 to 1,000,000	Signer, Caspersson, Hammarsten (366)
Same material	X-ray analyses	500,000 to 1,000,000	Astbury, Bell (12)
Desoxyribonucleic acid, thymus (cattle)	Ultracentrifuge	1,000,000 to 2,000,000	Schmidt, Pickels, Levene (351)
Desoxyribonucleic acid, thymus	Ultracentrifuge, diffusion	500,000	Tennent, Vilbrandt (396,408)
Desoxyribonucleic acid, thymus	Diffusion	1,500,000	Kahler (197)
Ribonucleic acid, tobacco mosaic virus, separated by:	Electrophoresis, ultracentrifuge, diffusion		Cohen, Stanley (70)
0.1 N NaCl, 100°, 1 min.		50,000 to 290,000	
Cold alkali		59,000 to 70,000	
Cold alkali, stored a few days, 4°, pH 7		15,000	
Ribonucleic acid, tobacco mosaic virus separated by alkali	Diffusion	37,000	Loring (265)
Ribonucleic acid, yeast, partly decomp.	Diffusion	17,000	Loring (265)
Ribonucleic acid, yeast, commercial	Diffusion	10,000	Fischer, Lehmann-Echternacht, Böttger (128)
Ribonucleic acid, commercial		10,000 to 23,000	Fletcher, Gulland (131)

ordinary, much investigated polynucleotides from yeast, wheat germs, and thymus gland contain only four bases in apparently uniform concentration (241,311). With modern analytical techniques, deviations from the equimolar distribution of the bases have been found, but they are not very striking in some instances. The struggle for some formulation of the facts at hand combined with a human weakness for simplification may account for the tetranucleotide theory. A very emphatic stand against the tetranucleotide theory has been taken recently by Gulland and his group (78,139,145). Other laboratories, notably those headed by Bredereck and by Fischer have voiced a more conservative opinion (41,42,125). Perusal of the early literature shows that considerable material can be found which fits well into the modern theory, and reference should be made particularly to the investigations of Hammarsten and Jorpes (157,160) and of Jorpes (193,194,245,298) on pancreas ribonucleic acid, which contains a large excess of guanine.

The most significant problems in relation to the structure of polynucleotides are the molecular weights, the type of linkage between the mononucleotides, formation of oligonucleotides (tetranucleotides?) in the course of chemical and enzymic degradation and the ratio of structural units in nucleic acids. These questions will be taken up presently. For the following discussion it should be kept in mind that much controversy in the past has resulted from the assumption that there is only one type of molecular architecture of ribonucleic acid, and only one way in which desoxyribose nucleotides are combined as polynucleotides. Since this is rather doubtful, any statement concerning the structure of a polynucleotide should be specified by a description of its origin and the procedure of isolation. Conflicting reports may often be due to the fact that the authors inadvertently compare nucleic acids which perhaps differ from each other as much as do starch, glycogen, and cellulose. It is a bad habit of some nucleotide chemists to use ill-defined polynucleotides in reporting even such delicate work as the isolation of split products and their titration. Papers suffering from such shortcomings deserve only limited consideration.

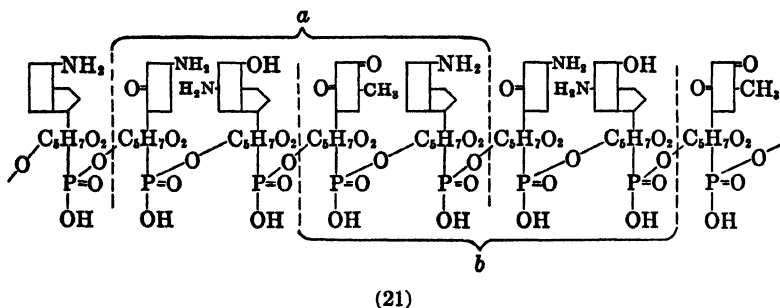
#### A. MOLECULAR WEIGHTS

Table IV shows the molecular weights of some desoxyribo- and ribonucleic acids, as recorded by various investigators. It is clear

from the data that both types are sensitive and that considerable breakdown may occur during isolation unless precautions are taken. To avoid decomposition, separation of the nucleic acids from the source material in form of the nucleoproteins has become customary (66,148,158,288,289,361,367,394) in the case of desoxyribonucleic acids; this is also possible with ribonucleic acids, particularly those combined with virus proteins (70,378). Regardless of what molecular weights of various specimens may eventually be accepted, it is already abundantly clear that nucleic acids in the native state belong to the class of high polymers, and the methods of investigation have to be chosen accordingly.

### B. TITRATION EXPERIMENTS

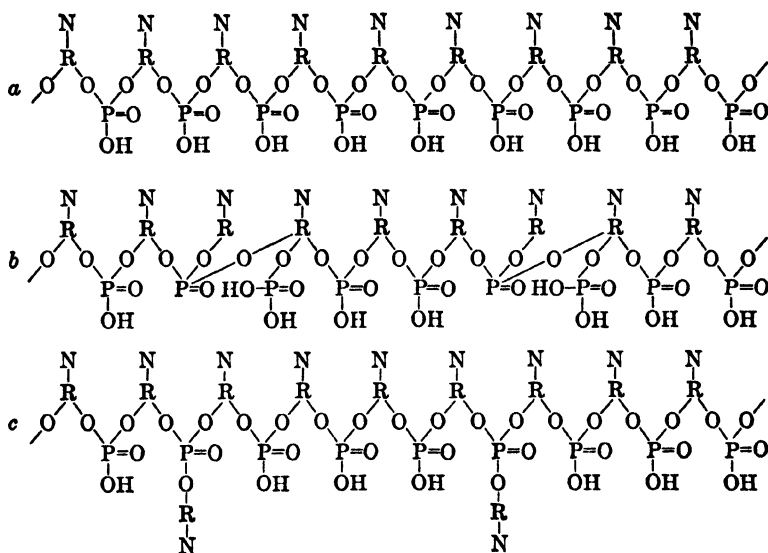
If nucleic acids consist of long chain molecules in which the carbohydrate units are connected by phosphoric acid (see formula 21, a suggested structure for desoxyribonucleic acid), for each base and carbohydrate phosphoric acid unit present in the molecule, one acid group should be titratable. The terminal phosphoric acid molecules showing two acid groups would not be numerous enough in native nucleic acids to alter the picture. The titration data of



Gulland, Jordan, and Taylor on desoxyribonucleic acid from thymus are in agreement with this concept (147). It is obvious that damage to the molecule in the course of the isolation procedure will create two additional acid groups for each split. This perhaps affords an explanation of many conflicting titration results in the past.

In ribonucleic acid from yeast, conditions were found more complicated. Titration data compatible with a straight chain structure similar to that given in formula (22a) were reported by some authors

(249,432), but Fletcher, Gulland, and Jordan noted that one out of every four phosphoryl groups titrated like a secondary dissociation (130,146). Instead of a straight chain structure an irregularity may be assumed as given in formula (22b). A further complication comes from observations that in some samples of ribonucleic acid there is a deficit of phosphoric acid groups amounting at times to as much as 25%. In formula (22c), a structure is given which would



(22)

account for this. In cases of a smaller deficiency in phosphoric acid, the side chains would occur less frequently in the molecule. A combination of the two types of structure may fit the titration data, and any reasonable proportion of primary and secondary phosphoric acid groups or deficit of phosphoric acid may be accounted for in this way by suitable variations.

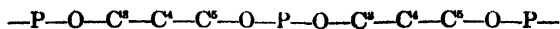
It would seem unwarranted to give configurations like those in formulas (22) merely to explain titration data. There are, however, other indications which favor the assumption of a more irregular structure of ribonucleic acid from yeast in contrast to desoxyribo-

nucleic acid. In their X-ray studies on ribonucleic acid Astbury and Bell found it difficult to get as satisfactory diffraction patterns as are obtained with desoxyribonucleic acid (10,11). The action of the enzyme ribonuclease is also irregular and does not yield uniform fragments from ribonucleic acid (see Sect. VII. F.). A sequence of nucleotides linked in uniform fashion as shown in formula (22a) would not offer an explanation for the isolation of split products of widely varying size after action of this enzyme. The peculiar situation is that we can develop a more satisfactory picture of the desoxyribonucleic acid than of the ribonucleic acid molecule, in spite of the far more advanced knowledge on the structural units of the latter.

### C. MOLECULAR ARCHITECTURE OF NUCLEIC ACIDS

Studies of the flow properties of sodium desoxyribonucleate from thymus led Signer, Caspersson, and Hammarsten to the conclusion that the molecule is about 300 times as long as it is wide (70,366, 381). X-ray studies by Astbury and co-workers (10) have given a more detailed picture. The average thickness of the mononucleotides in the chain was found to be 3.3 to 3.4 Å. Great importance is attributed to a regular, recurring arrangement along the fiber axis, the period consisting of at least eight or sixteen nucleotides. The fact that the intramolecular pattern is found to be based on a multiple of four nucleotides is believed to have significance, and from the perfection of the X-ray fiber diagram Astbury is inclined to assume that the four different kinds of desoxyribose nucleotides cannot be distributed simply at random; they must follow one another in some definite order. It is interesting to see these physico-chemical studies favoring a concept which has been so readily discarded by many biochemists.

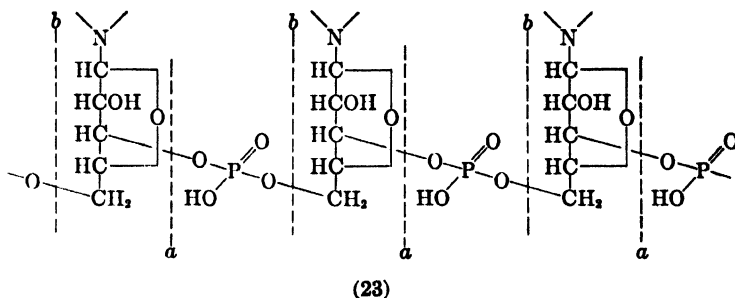
The dense packing of the mononucleotides makes necessary the assumption that the purine and pyrimidine rings are arranged flat upon each other, and the carbohydrate rings are organized in the same fashion, but not coplanar with the bases, since base and sugar are linked at an angle. The structure may be visualized by assuming leaves on one side of a stem in close succession. The spine of the molecule may consist of the grouping:



In this sequence, carbon atoms 3, 4, and 5 belong to the carbohydrate and the atoms are linked at angles so that only the over-all extension of this "back bone" of the nucleic acid molecule is straight (10). It has not yet been possible to develop so detailed a picture of the structure of ribonucleic acid by X-ray studies, but there are indications that some similarity may exist (10). Stern has developed interesting models visualizing nucleoprotein and nucleic acid molecules (196,360,381).

#### D. MODE OF LINKAGE OF THE NUCLEOTIDES IN NUCLEIC ACIDS

In ribonucleic acids the mononucleotides are presumably combined by phosphodiester linkages involving positions 3 and 5 of the sugar. The alternative would be position 2, since 1 and 4 are excluded by the furanoid ring structure. Esterification at carbon atoms 3 and 5 of the sugar, however, fits better into the molecular geometry, as



has been pointed out by Astbury (10). Unfortunately, it is difficult to reconcile the results of alkaline hydrolysis with this suggested structure. The mononucleotides obtained in good yield contain the phosphoric acid in position 3, indicating that the split *b* in formula (23) is greatly preferred to split *a*. Fission of type *a* would give 5'-phosphonucleosides, but they have never been found in the hydrolyzates of ribonucleic acids, nor have any diphosphoribose nucleotides been found. The 5-ester linkage should therefore be much more labile than the 3-ester linkage, but this is not so. Attachment of phosphoric acid in position 5 is known from the co-enzyme nucleotides, and in the most thoroughly studied of them,

adenosine-5'-phosphoric acid, the ester linkage is rather stable under conditions which split ribonucleic acid. Thus position 2 was considered instead of 5 as the point of connection. There is no reason, however, to assume great lability of ribose-2-phosphate compared to other ribose phosphates. Moreover, Gulland and Smith (150) synthesized uridine-2'-phosphoric acid and found its stability toward alkali of the same order as that of uridine-3'-phosphate isolated from nucleic acid. The nature of the principal internucleotide linkage, therefore, is a very unsettled question, and the picture is further complicated when the possible deviations from the regular structure (formulas 22) are taken into consideration. At present, the facile hydrolytic resolution of the internucleotide ester linkages can be explained only by assumption of physical factors like an intramolecular strain due to the dense packing of the structural units. Linkages between the ribose nucleotides other than phosphoric acid ester bridges appear improbable for a number of reasons. The nucleic acids can be deaminated without splitting the molecules into significantly smaller fragments (130). The isolation of guanine uridylic acid (39) has been explained by Bredereck as an artifact, while Gulland still maintains that this is a true cleavage product (120,138), which, however, cannot be isolated from all ribonucleic acids. Refined methods of electrometric titration account for all amino and hydroxy groups of the bases (130).

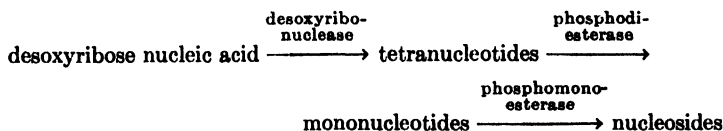
Concerning the internucleotide linkage of desoxyribonucleic acids much the same considerations seem to hold, but here the uncertainty is increased by the fact that the characterization of the structural units is not yet complete. Position 2 of the carbohydrate is excluded as the site of esterification provided the sugar is identical with D-2-desoxyribose in all nucleotides. On desoxyribonucleic acid from thymus Gulland and co-workers (147) obtained titration and viscosity data which suggest very labile linkages between amino and hydroxy groups of adjoining bases. However, since no exact statement about the type of bonds can be made so far, these linkages are not indicated in formula (21).

#### E. ENZYMIC DEGRADATION OF NUCLEIC ACIDS

One of the main questions concerning the enzymic as well as the chemical degradation of nucleic acids is the formation of well-defined and uniform oligonucleotides (tetranucleotides?). (The term

oligonucleotide—from *ὀλίγος* = small, few—has been coined by Fischer, Lehmann-Echternacht, and Böttger (128) to denote nucleic acid fragments larger than mononucleotides, but not larger than tetranucleotides.) A prerequisite for the appearance of such units would be uniform concentration and repetition of the four bases of each nucleic acid throughout the chain. This seems doubtful at least with respect to ribonucleic acids (see below, Sect. H.) and the existence of uniform ribose tetranucleotides as maintained by Brederick (41) is questionable. Desoxyribonucleic acid from thymus seems to offer a better chance of decomposition into tetranucleotides, because the bases occur apparently in equimolar concentration, and there is no evidence against a well-defined sequence as exemplified in formula (21). Still it is not conceivable that chemical fission should lead to tetranucleotide *a* (formula 21), for example, in preference to *b*. The bases do not seem sufficiently different to impress upon the molecule spots of lesser resistance at every fourth unit. Enzymic formation of tetranucleotides, on the other hand, appears more likely, since substrate specificity of desoxyribonuclease may restrict fission to an ester linkage adjoining one particular base.

The best evidence so far for the appearance of desoxyribose tetranucleotides in the course of enzymic degradation comes from the work of Lehmann-Echternacht (126,127,236). Adenine and thymine are supposed to be the corner stones of the molecule. A special enzyme (phosphodiesterase) is claimed for the formation of mononucleotides from the tetranucleotide. The process may be summarized by the following scheme:



It would seem that experimental approaches to the problem of nucleic acid structure by means of well-controlled action of highly purified or pure enzymes give more hope for results than any other method. Unfortunately, phosphatases in most instances show much lower substrate specificity than other groups of enzymes. Satisfactory separation of phosphomonoesterases from phosphodiesterases is difficult. Even the highly purified intestinal phosphatase is disappointing with respect to substrate specificity (352). As Schmidt



has pointed out (348) the wealth of information on enzymic phosphorylations and dephosphorylations concerns phosphoric acid monoesters, phosphoamides, or pyrophosphates, while little is known about the enzymes dealing with phosphoric acid diester linkages.

#### F. RIBONUCLEASE

Among the enzymes of nucleic acid metabolism ribonuclease is interesting in several ways. It can be obtained readily in crystalline form from pancreas (230). The molecular weight is remarkably small and has been reported between 13,000 and 15,000 (331). It is stable at pH below 5 at 100° for at least five minutes and one prosthetic group was found. Contrary to the history of most other enzymes, crystallization was accomplished in an early stage but, although nine years have elapsed since then, the exact function and the range of action of ribonuclease are still controversial (276a).

Ribonucleic acids are split by ribonuclease without formation of inorganic phosphate into small fragments which, in contrast to the starting material, are readily diffusible (64,230,305,355). The process is not quantitative, however, and the degree of splitting depends on the type of ribonucleic acid. Loring and Carpenter found adenylic, guanylic, cytidylic, and uridylic acid among the reaction products (267), but such completeness of action is not substantiated by other investigators. It appears rather that oligonucleotides are formed which are small enough to diffuse readily through membranes, and, in contrast to ribonucleic acid, are not precipitated by strong acetic acid or uranyl acetate and trichloroacetic acid. Complete splitting into mononucleotides would approximately double the number of titratable groups, but the observed values remain far behind theory when the amount of diffusible material is taken as a basis of calculation. It seems justified, therefore, to assume formation of split products much smaller than the original nucleic acid, but on the average larger than mononucleotides. The digestion cannot be brought to completeness, apparently, and the observed limits are 50 to 85% splitting, depending on the material.

A variety of conditions may be responsible for the divergent reports on ribonuclease action. First, the multiple nature of ribonucleic acid should be kept in mind. The difference may be due to the source of the substrate and the degree of alteration during the isolation procedure. Commercial preparations sometimes contain mono-

nucleotides and heavy metal contaminations which may be inhibitory (431,434). The variability of ribonuclease action and the influence of the ionic milieu command caution in its use for quantitative measurements (136).

Important progress in tracing the action of this enzyme has been made recently by Schmidt, Cubiles, Swartz, and Thannhauser (347). They observed that prostate phosphatase can liberate phosphate quantitatively from mononucleotides, but not from ribonucleic acid or split products larger than mononucleotides. Incubation with ribonuclease prior to prostate phosphatase action renders 25% of the total phosphate susceptible to the latter. This mineralized phosphoric acid fraction comes from a pyrimidine nucleotide.

#### G. DESOXYRIBONUCLEASE

Many of the statements about the enzymic degradation of ribonucleic acids hold for desoxyribonucleic acids as well. Desoxyribonuclease action is the first step and the enzyme bringing about this reaction is not identical with ribonuclease (215,236,305). Considerable purification has been brought about by Klein (217), and McCarty (276), and its isolation in the crystalline state from beef pancreas has been announced recently by Kunitz (231). This accomplishment offers the prospect of elucidating the mode of action and the range of this enzyme.

Zamenhof and Chargaff (430a) have obtained another desoxyribonuclease from yeast by extraction with sodium chloride solution. This enzyme is combined with an inhibitor. It is slowly released on storage at low temperature, resulting in about a fiftyfold increase of activity after three months. The enzyme resembles pancreas desoxyribonuclease in many respects, but it is insoluble in water. The inhibitor does not interfere with the activity of Kunitz's enzyme from pancreas (430a).

According to Fischer, Lehmann-Echternacht, and Böttger (128, 236), one acid equivalent for each four molecules of phosphoric acid is produced by desoxyribonuclease. This would seem to indicate formation of tetranucleotides, as discussed on page 517.

#### H. IRREGULARITIES IN THE COMPOSITION OF NUCLEIC ACIDS

The structural considerations on the preceding pages are based on the assumption that all ribonucleic and desoxyribonucleic acids

regardless of origin are built essentially in one fashion. Substantial proof for this is lacking, but it may be advantageous at the present time to search for the more general principles of structure which these acids have in common, and to set aside elucidation of individual deviations from the main pattern until a firmer foundation for the formulation of nucleic acid structure is established. Some indications of individuality, however, are already claimed. The peculiarity may involve the internucleotide linkage; the isolation of an isomeric cytidylic acid from a tobacco mosaic virus seemed an example of an altered type of ester linkage within a mononucleotide (266). This claim has not been maintained (359a). The deficit of phosphoric acid in some polynucleotides has been stressed; on the other hand, the equimolar ratio between carbohydrate and bases has not so far been questioned. Several investigations deal with the relative amounts of bases occurring in nucleic acids. It seems that equimolarity of them does not hold for a number of specimens. The outstanding and well-established example is the ribonucleic acid from pancreas studied by Jorpes (193,195,245), which has an excess of one to three moles of guanine over the other bases. More experimentation is required before we may claim that variable distribution of purines and pyrimidines is the rule rather than the exception. The work so far pertains to ribonucleic acids primarily, while deviations from uniformity in base distribution have been claimed only rarely (138) for desoxyribonucleic acids. This, however, may reflect merely the more limited number of samples scrutinized so far.

Any experimental approach to quantitative analysis of the structural units of nucleic acids is difficult. The limitations of elementary analyses are apparent from Table V, in which the compositions of the constituent nucleotides of both nucleic acid types are listed. The values are proportionately lower when the units are combined to form nucleic acids by elimination of one molecule of water for each internucleotide bond. Comparison of the data with the average composition shows that elementary analyses might reveal only gross anomalies which would be indicated mainly by a change of the nitrogen values.

Chemical separation of the bases by utilizing the difference in stability of the purine and pyrimidine glycoside bonds has been widely used (195,241,347). The procedures, however, are cumbersome, and failure to find equimolar ratio of the bases does not in

itself justify the claim that this represents an anomaly in the nucleic acid examined. It would be desirable, at least, to check the reliability of the analytical procedure on a nucleic acid sample of widely different origin. Dissimilarity of the results would speak in favor of a true difference in composition of the two samples. Model experiments with known mixtures of a few bases do not seem sufficient in view of the numerous pitfalls of nucleic acid analysis. There is room for improvement, and the modern tools of quantitative biochemistry have not been exhausted. In particular, the dilution technique with labeled compounds, so successfully used in protein analysis, has not yet been applied to the nucleic acid field.

TABLE V  
ELEMENTARY COMPOSITION<sup>a</sup> OF RIBOSE AND DESOXYRIBOSE NUCLEOTIDES

Acid <sup>b</sup>	C	H	N	O	P
Adenylic C <sub>10</sub> H <sub>14</sub> N <sub>5</sub> O <sub>7</sub> P (347.24)	34.59	4.06	20.17	32.25	8.92
Guanylic C <sub>10</sub> H <sub>14</sub> N <sub>5</sub> O <sub>8</sub> P (363.24)	33.06	3.88	19.28	35.24	8.53
Cytidylic C <sub>9</sub> H <sub>14</sub> N <sub>4</sub> O <sub>8</sub> P (323.21)	33.44	4.37	13.01	39.60	9.58
Uridylic C <sub>8</sub> H <sub>13</sub> N <sub>2</sub> O <sub>8</sub> P (324.19)	33.34	4.04	8.64	44.42	9.56
<i>Average</i>	<i>33.61</i>	<i>4.09</i>	<i>15.28</i>	<i>37.88</i>	<i>9.15</i>
Desoxyadenylic C <sub>10</sub> H <sub>14</sub> N <sub>5</sub> O <sub>6</sub> P (331.24)	36.26	4.26	21.14	28.98	9.35
Desoxyguanylic C <sub>10</sub> H <sub>14</sub> N <sub>5</sub> O <sub>7</sub> P (347.24)	34.59	4.06	20.17	32.25	8.92
Desoxycytidylic C <sub>9</sub> H <sub>14</sub> N <sub>4</sub> O <sub>7</sub> P (307.21)	35.18	4.59	13.68	36.46	10.08
Thymidine phosphoric C <sub>10</sub> H <sub>15</sub> N <sub>2</sub> O <sub>8</sub> P (322.22)	37.27	4.69	8.70	39.72	9.62
<i>Average</i>	<i>35.83</i>	<i>4.40</i>	<i>15.92</i>	<i>34.35</i>	<i>9.49</i>

<sup>a</sup> In per cent.

<sup>b</sup> Molecular weights are given in parentheses.

Very promising bioassays have been initiated by Loring and his colleagues (268,270) with *Neurospora* mutants. One of them uses only pyrimidine ribonucleosides or nucleotides, while another requires adenine (290). The mold does not distinguish between the cytosine and uracil nucleosides or nucleotides, which, by the way, provides evidence of their interconversion *in vivo*. Several specimens of ribonucleic acid were analyzed with *Neurospora* mutants. The samples were prepared for bioassay in the following way. For pyrimidine nucleoside determination, hydrolysis was carried out in 2.5% ammonia at 140 to 150° for four hours. This was followed by

a chemical separation using phosphotungstic acid. Only cytidine and cytidylic acid are brought down by this reagent, while uridine and uridylic acid remain in solution. Refluxing with 1 *N* sulfuric

TABLE VI  
PYRIMIDINE NUCLEOSIDES AND NUCLEOTIDES, ADENINE, AND GUANINE CONTENTS  
OF DIFFERENT RIBONUCLEIC ACID PREPARATIONS (270)

Sample	Phosphorus, %	Total pyrimidine nucleoside*	Uridine*	Total pyrimidine nucleotide*	Adenine (or hypoxanthine)*	Guanine*
Yeast nucleic acid (Lemke 5897)	8.77	0.385	0.21	—	—	—
Yeast nucleic acid (Schwarz A 315-31414)	8.27	0.37	0.20	—	—	—
Yeast nucleic acid (Schwarz HN 4625)	8.82	—	—	0.259	0.59	0.30
Tobacco mosaic nucleic acid	8.23	0.363	0.175	0.392	0.60	0.29
Purified yeast nucleic acid (acetic acid insoluble)	7.78	0.366	0.179	0.334 <sup>b</sup>	0.16 <sup>c</sup>	0.31
Purified yeast nucleic acid (acetic acid-alcohol insoluble)	8.3	—	—	—	0.53	0.27
Ribonuclease-resistant fraction (acetic acid insoluble)	7.8	<0.016	—	0.16	0.53	0.49
Ribonuclease-resistant fraction (acetic acid-alcohol insoluble)	7.9	<0.016	—	0.16	0.85	0.31
"Statistical" tetranucleotide	9.52	0.50	0.25	0.50	0.25	0.25

\* In moles per mole of phosphoric acid.

<sup>b</sup> After four-hour hydrolysis with 0.4 *N* triethylamine.

<sup>c</sup> Calculated as difference between total purine and guanine.

acid for one hour yielded the purine bases and left the pyrimidine nucleotides intact. Guanine was determined by the chemical method of Hitchings (171). The results of this work are shown in Table VI. Obviously, the reliability of the data depends largely on the course

of the hydrolyses and fractionations, and some uncertainty may attend the assay method (269,269a, 316a). Nevertheless, the error involved would concern every nucleic acid fraction in identical fashion; discrepancies between various preparations, therefore, remain significant. There is no doubt that marked deviations in the distribution of the bases from the statistical tetranucleotide values are apparent. Interesting is the low pyrimidine content of the ribonuclease-resistant fraction, which may be taken as an indication that this enzyme in preference liberates pyrimidine nucleotides from the polynucleotide chain, as mentioned earlier.

Vischer and Chargaff (409) have extended the knowledge of irregularities in the distribution of the bases. Their elegant technique of paper chromatography combined with spectrophotometry seems to provide an entirely new basis for quantitative analysis of nucleic acids. Very little uracil was found by the authors in ribonucleic acid from yeast and from pancreas, while desoxyribonucleic acid from thymus and spleen appears to be deficient in cytosine (65a).

### VIII. Outlook

Despite the increased interest in the chemistry and enzymology of nucleic acids the research activity in this section of the field is lagging conspicuously behind the study and the endeavor which biologists of various denominations have given to this class of compounds. The chemistry of nucleic acids is not even sufficiently advanced to have established the nature of the internucleotide linkage which is an equivalent of the peptide bond of protein chemistry. The reason for this situation may be partly an outgrowth of the historical development of the subject. Nucleic acids have been the realm of biologists since their discovery. With few exceptions chemists of the past were deterred from concerning themselves with a group of compounds so complex and so difficult to handle when countless other problems offered more immediate reward at the expenditure of less time and ingenuity. Also until recently, none but the simplest units of the nucleic acid realm permitted confirmation of suggested structures by synthesis, which is one of the ambitions of every true organic chemist. Enzymological studies were hampered for a long time by the fact that well-defined substrates (nucleosides, nucleotides, and polynucleotides) were not readily available. Em-

barking on a research program along these lines made necessary the cumbersome preliminary of preparing adequate amounts of these substances. There has been some notable change in this respect recently, and it may be expected that in the near future even desoxyribose nucleosides and nucleotides will be more readily accessible for these investigations. The number of major laboratories emphasizing work of the type reviewed here has always been limited. A serious decrease is due to the unfortunate circumstances of recent years. But the amazing volume of biological investigations appearing now, no doubt, will cause a surge of chemical and enzymological work in coming years. There are numerous problems within reach of the individual investigator which do not require expensive equipment or years of tedious apprenticeship.

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# PATHWAYS OF ACID FORMATION IN ASPERGILLUS NIGER AND IN RELATED MOLDS

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## I. Introduction

Citric acid was isolated for the first time by Scheele, who, in 1784, obtained it from the juice of lemons. It is distributed widely in nature for, with the exception of cherries and plums, almost all fruits contain some citric acid, and it is often found in association with one or more of the four-carbon dicarboxylic acids and with oxalic acid. More rarely, tricarballic, isocitric, and aconitic acids,

all of which are closely related to citric acid, have been found in plants. Interest in citric acid was enhanced in 1891 by Wehmer's discovery (150) that it is formed by a type of fungus which he termed "*Citromyces*." This has been compared to *Aspergillus* and *Penicillium* (125) and a later authority (140) decided that the characteristics of *Citromyces* are insufficient to distinguish it from the aspergilli. The yields obtained by Wehmer were sufficiently encouraging to lead to intensive efforts to devise industrial processes for the mycological production of citric acid, but real success did not attend these efforts until the investigations of Currie (50) had indicated the principal factors which govern the production of citric acid in high yield in cultures of *A. niger* on sugar. Following Currie, most of the processes for the technical production of citric acid by mycological agency have been based upon the use of *A. niger*.

Citric acid occurs not only in cultures of species of *Aspergillus* and *Penicillium* but has also been isolated from among the metabolic products of molds belonging to other genera, for example, some strains of *Botrytis cinerea* can form it from sugar, and Wehmer detected it in cultures of *Mucor piriformis* Fischer.

Citric acid is very rarely, if ever, the sole acidic product of the action of *A. niger* on sugar, for gluconic acid and oxalic acid are also formed by the majority of strains and, in the different cases, their relative proportions may be very different; in fact enormous variations occur not only in the citric-acid-forming capabilities of different strains of *A. niger* but also in the respective abilities of these strains to form gluconic and oxalic acids.

One strain may give gluconic and citric acids in good yields and very little oxalic acid; another may afford only small quantities of the two first-mentioned acids but give much oxalic acid. Strains of *A. niger* used by Currie were stated by him to yield no gluconic acid at any stage of the process, the acid product being found to consist solely of citric acid, though sometimes it was noted that a little oxalic acid made its appearance later when the mycelia were aging. Usually, it has been found that *Penicillium* species produce chiefly gluconic and citric acids, and that the *Mucor* species give rise to succinic, fumaric, and oxalic acids.

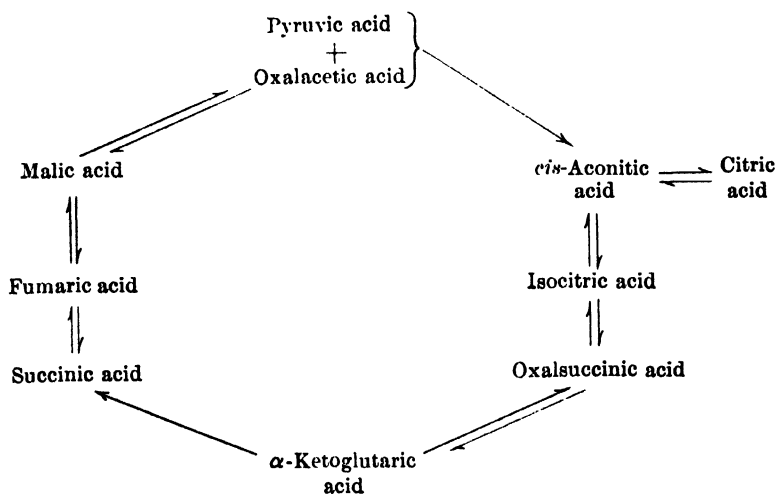
Strains of *A. niger* which, when first isolated from natural sources, prove to be efficient producers of acid can usually be maintained at that level of efficiency by providing them with the requisite nu-

trients and conditions for growth. In the event of a falling-off in acid-forming ability, due to unsuitable nutritional conditions, a regeneration treatment can be applied and sometimes is successful in restoring acid-forming power (41a). On the other hand, strains which are poor producers of acid when first isolated are found to remain so, and no treatment can improve them. Sometimes acid-forming capability changes for no apparent reason, or the nature of the product is altered; thus a strain may cease to yield citric acid and give instead only gluconic acid. When such a change is found to be irreversible it is possible that mutation has occurred, though the possibility that a contaminant of similar appearance has gained access to the culture and overgrown the original strain cannot always be ruled out. Yuill and Yuill (166) reported a most interesting case of this type. They isolated from cultures of *A. niger* a new fungus and its morphology was seen to be similar to that of *A. niger* until after the production of sterigmata, when its development followed an entirely different course. This necessitated the provision of a new form-genus, for which they proposed the name *Cladosarum*. The morphological differences between *Cladosarum* and *A. niger* go far beyond those associated with previously described mutants. The new organism showed to a striking extent the biochemical activities of *A. niger*. The authors considered that its origin from *A. niger* was not improbable.

Thus it can readily be realized that, in working industrial processes founded upon the use of mold fungi, strict control of the stock cultures as well as attention to the general cultural conditions is an unceasing obligation; it is this fact which renders the control of such processes a more difficult matter than, for example, the production of industrial alcohol by means of yeast, for *Saccharomyces cerevisiae* maintains relatively a far greater uniformity of behavior than do the various species of acid-forming molds.

Molliard (105) found that low concentrations of nutrient salts, with abnormally small quantities of inorganically combined nitrogen, favored formation of gluconic acid and depressed that of citric acid in glucose cultures of *A. niger*. Somewhat similar results were obtained by Bernhauer (9), who observed that, up to certain respective limits, increase of nitrogen source and of temperature resulted in increase in yield of citric acid and in the formation of negligibly small quantities of gluconic acid.

Consideration of the experimental findings of Currie (50), of Molliard (105) and of other workers makes it evident that when more than one acid is produced by *A. niger* the maximum concentrations of the several acidic products are reached at different times. Usually there is first formation of gluconic acid, the concentration of which, after rising, falls off while citric acid accumulates. Later, the citric acid diminishes while oxalic acid accumulates. Finally, only oxalic acid may be left and this also in turn is gradually metabolized. These changes are all rather gradual and the three acids may be and, in fact, often are coexistent in the medium, but, as indicated, their proportions constantly change. Citric acid can be



SCHEME I

formed not only from glucose and from sucrose but also from certain sugars and sugar alcohols possessing three, five, or six atoms of carbon (glycerol, glycerose, xylose, arabinose, fructose, galactose, mannose, and mannitol). Amelung (2), who investigated this subject, stated that sugars or their derivatives with four and with seven carbon atoms (erythritol and glucoheptose) gave no citric acid. He found that gluconic acid could only be obtained from glucose, sucrose, and maltose, and thought it improbable that gluconic acid

could be a precursor of citric acid, since the yields were low when the former acid was used as substrate. All these facts show the complexity of the biochemical problem presented by citric acid formation in molds.

In recent years studies of intermediary metabolism in the animal body have revealed that in pigeon liver, in pigeon breast muscle, and in sheep's heart, citric acid arises by a side reaction from *cis*-aconitic acid. The latter in this connection fills the role of an essential metabolite participating in Krebs' tricarboxylic acid cycle (Scheme I) in these tissues. The question therefore arises whether formation of citric acid in molds is brought about by the same sequence of reactions as those which lead up to Krebs' cycle in certain animal tissues. It will be one of the purposes of this article to set out and to examine such evidence bearing on this question as is now available, and to endeavor to decide how far the information takes us along the road to a solution of the problem. A critical survey of such evidence will necessarily involve consideration of the mechanisms by which acids other than citric acid arise during metabolic processes in mold fungi. Finally, since the publications to which reference will be made are spaced over a fairly long period, the presentation in the earlier sections will show the historical development of the inquiries. In later sections reference will be made to the results of investigations, not yet published in detail, which have been undertaken recently in the writer's laboratory. Acknowledgments are due to Prof. F. Challenger who kindly supplied the subject matter of the section dealing with kojic acid.

## II. Early Views on the Mechanism of Formation of Citric Acid

Examination of the various hypotheses formulated in explanation of the mycological conversion of the straight chain form of glucose into this six-carbon acid possessing a branched chain shows that the chemical changes involved in the transformation have been regarded, by different workers, from very diverse points of view. The hypothesis of Claisen and Hori (44) appears to be the earliest suggestion respecting the mode of formation of citric acid in nature. Having synthesized aconitic acid by processes starting from the condensation of ethyl acetate with ethyl oxalate, these authors



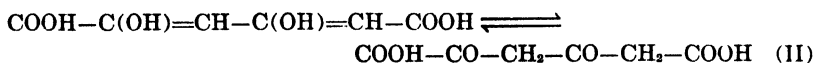
considered that citric acid in plants might have its genesis in an analogous condensation of 2 molecules of oxalic acid with 2 molecules of acetic acid, yielding (I), which would be an oxalyl derivative of aconitic acid:



It was presumed that (I) would then split to oxalic acid and aconitic acid and the latter by addition of water would yield citric acid. There is nothing in this scheme which can be considered to be at variance with the known facts of organic chemistry.

Kostychev and Chesnokov (86) believed citric acid to be a by-product of protein synthesis from sugar, but this view cannot explain the very high yields (ca. 89 to 90%) of citric acid which, it is now known, can be obtained by the action of some molds.

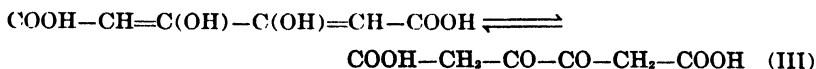
Raistrick and Clark (121) brought to bear on the question views derived from Collie's publications (46) on the polyketides. These ideas, together with the observation of Voisenet (146) that *Bacillus amaracrylus* produces acrolein from glycerol, led them to suggest that were the molecule of glucose to suffer a similar loss of the elements of 2 molecules of water, together with oxidation at the terminal carbon atoms, a very reactive compound would be formed. Such a compound might be  $\alpha,\gamma$ -diketoadipic acid (II) which, on hydrolysis, should split readily into acetic and oxalacetic acids. An



aldol condensation between the two last-mentioned acids might then be presumed to yield citric acid. The suggestions of Raistrick and Clark also offered an explanation of the formation of the oxalic acid which, in cultures of *Aspergillus niger*, often accompanies or follows that of citric acid, for they believed the former acid to be produced from acetic acid by oxidation and from oxalacetic acid by hydrolytic fission, which would give rise to oxalic acid plus acetic acid. The authors obtained oxalic acid in yields of 39 to 40% when *A. niger* was grown on sodium acetate, but they offered no experimental evidence of the presence of oxalacetic acid or of acetic acid in cultures of *A. niger*. They quoted the experiments of Pfeffer (118) as indicating that there is little likelihood of detecting acetic acid in cultures of *A. niger* on sugar, for Pfeffer had shown

that calcium acetate is decomposed at least as rapidly as glucose when both together are submitted to the action of the mold.

Challenger, Subramaniam, and Walker (38) drew attention to a suggestion of Franzen and Schmitt (61) to the effect that, since the ethyl ester of  $\beta,\gamma$ -diketoadipic acid (III) undergoes a "benzilic acid transformation" in the presence of alkali metal hydroxide,

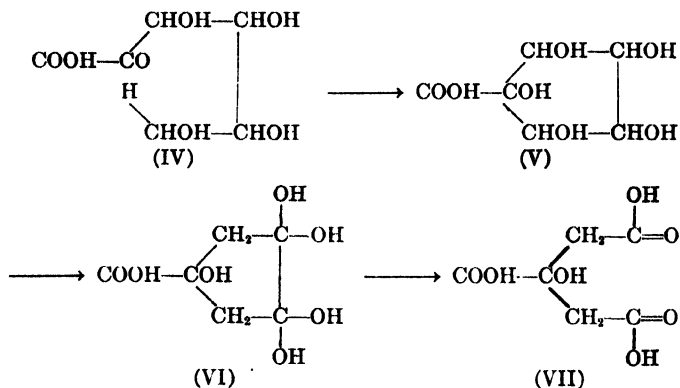


yielding ethyl citrate, the biological formation of citric acid may be effected in an analogous manner, the diketo acid supposedly arising from saccharic acid. Challenger *et al.* detected small amounts of saccharic acid in cultures of one strain of *A. niger* on glucose and also on calcium gluconate and they showed also that citric acid could be isolated in small yield from cultures of the same mold on salts of saccharic acid. In later experiments, however, in which other strains of *A. niger* were employed, these results could not be repeated; this was also the experience of Bernhauer (15), so it must be considered improbable that saccharic acid is an intermediate in the mycological formation of citric acid from sugar.

The idea of Euler (55) that 3 molecules of acetaldehyde arising from sugar might give rise to the dialdehyde of  $\beta$ -methylglutaric acid which, he suggested, would give citric acid on oxidation, presumes behavior that has not been observed *in vitro*; similar absence of experimental support is associated with the suggestion of Buchner and Wüstenfeld (22) that conversion of glucose to citric acid could proceed *via* a compound analogous to the parasaccharinic acid obtained from lactose by treatment with lime water.

Finally, we come to the interesting suggestion of Butkevich (24), who considered that the aldehyde form of glucose might undergo an internal aldol condensation yielding a cyclic polyhydroxy compound which, by loss of water, followed by oxidative disruption of the ring, might be presumed to afford citric acid. Later, when it had been shown (19,147) that strains of the *A. flavus oryzae* group and *A. parasiticus* Speare contain an enzyme capable of converting glucose to glucosone, Butkevich and co-workers (30) quoted this fact as support for the view that formation of a polyhydroxy cyclic compound is necessary for citric acid formation, for they considered that D-2-ketogluconic acid, derived perhaps from glu-

cosone, might well undergo ring formation in accordance with Butkevich's hypothesis, as shown in the sequence (IV) to (VII):



These authors also entertained the view that cyclization followed by a similar series of changes leading to citric acid might occur in D-glucuronic acid or in D-aldehydogleuconic acid. The last-mentioned acid is produced from glucose by certain species of *Acetobacter* (137), while the formation of D-glucuronic acid in cultures of the fungus *Ustilina vulgaris* on a glucose medium has been reported by Wünschendorff and Killian (164). This fungus can also form citric acid. Butkevich also quotes a reference to the formation of large quantities of glucuronic acid in moldy grapes.

Using the sodium salt of glucuronic acid as substrate Butkevich *et al.* were unable, however, to detect the formation of citric acid and they concluded that in glucose cultures of *A. niger* the latter acid arises most probably by way of D-2-ketogluconic acid. They failed, however, to furnish any experimental evidence in favor of this conclusion.

Molliard (105), Bernhauer (8), and Butkevich (25) independently detected gluconic acid at an early stage in the development of *A. niger* on sugar media, but they were unable to show that gluconic acid yields citric acid under the influence of the mold. This was demonstrated, however, by Wehmer (151) and by Schreyer (129) using salts of gluconic acid as sole source of energy, but the yields were not high and neither considered gluconic acid to represent an essential stage in the path from sugar to citric acid.

### III. Zymasis in Relation to Acid Metabolism

#### A. OCCURRENCE OF ZYMASIS IN MOLDS

The hypothesis of Raistrick and Clark that acetic and oxalacetic acids are the immediate precursors of citric acid, in the mycological production of the latter from sugar, had not been substantiated by the isolation of presumed intermediates and interest became centered in the possibility that a different sequence of reactions, involving the intermediate formation of acetaldehyde or ethyl alcohol, might account for the observed facts relating to the production of citric and oxalic acids.

The formation of citric acid from ethyl alcohol by mycological action was observed first by Mazé and Perrier (103) and later it was realized that many strains of *Penicillium* and of *Aspergillus* under suitable conditions can form appreciable quantities of alcohol from sugar. Kostychev (84), in experiments on the anaerobic metabolism of *A. niger*, obtained glycerol, alcohol, and carbon dioxide in the proportions realized in fermentation by yeast, and later the same behavior was noted by Butkevich (23) in work with *Mucor* cultures.

Butkevich considered the acid-forming processes in *Mucor* species not to be closely related to those which lead to alcohol, since he observed that those species of *Penicillium* and of *Aspergillus* which are typical producers of citric acid give rise simultaneously to little or no ethyl alcohol, while those *Mucor* species, e.g., *M. racemosus*, which more readily form ethyl alcohol from sugar, are incapable of also giving as end products more than traces of acids.

#### B. FORMATION OF SUCCINATE AND FUMARATE FROM ACETATE AND FROM ALCOHOL

Later experiments of Butkevich and Fedorov (27) afforded evidence opposed to the original view of the former that alcohol formation is not closely related to formation of acids in molds. Various species of *Mucor* produced succinic acid and fumaric acid when supplied only with acetates or with alcohol as carbon source. *M. stolonifer* accumulated calcium fumarate (87% of the total product being fumaric acid and 13% being succinic acid) when grown on sugar cultures containing calcium carbonate. When acetate was supplied in place of sugar, the main product was succinate, together

with a small quantity of fumarate. When alcohol was supplied as sole source of carbon, fumaric acid was formed, together with traces only of succinic acid. The action of the mold on succinate alone gave much calcium oxalate and only traces of calcium fumarate. It would seem that the conversion of succinate to fumarate is greatly assisted by the presence of certain products of glycolysis, which may serve as acceptors of hydrogen. From consideration of these results Butkevich and Federov formed the opinion that in sugar cultures of the mold part of the sugar is broken down through the operation of the reactions of alcoholic fermentation, yielding glycerol, ethyl alcohol, and carbon dioxide and that succinic acid is then formed by a Thunberg dehydrogenation of acetic acid derived by oxidation of acetaldehyde or alcohol. Similarly, dehydrogenation of succinic acid could give fumaric acid.

Kostychev and Frey (85) established the fact that in yeast fermentations in presence of calcium carbonate organic acids are formed from some of the alcohol and Lundsgaard (96) observed that, during the respiration of yeast cells supplied only with alcohol, a large part of the alcohol oxidized is converted to organic acids. Carbohydrate (glycogen) is also synthesized from some of the alcohol. From the observed quantity of oxygen required to oxidize the alcohol it could be concluded that the acid formed was a mixture of succinic, fumaric, and malic acids. These findings are thus seen to fall in line with those of Butkevich and Fedorov in their experiments with *M. stolonifer*.

#### C. FORMATION OF GLYCOLATE FROM ACETATE AND OF MALATE FROM SUCCINATE

Prior to the work of Butkevich and Fedorov it had been shown by Challenger, Subramaniam, and Walker (38) that glycolic acid may be obtained as one of the products of the action of *A. niger* on calcium acetate. Also, Challenger and Klein (36) had isolated L-malic acid from cultures of the same mold on a fumarate medium, while Stent, Subramaniam, and Walker (134), by utilizing strains of *A. niger*, had been able to convert succinic acid to a mixture of the L and DL forms of malic acid.

#### D. FORMATION OF CITRATE FROM ACETATE AND FROM ALCOHOL

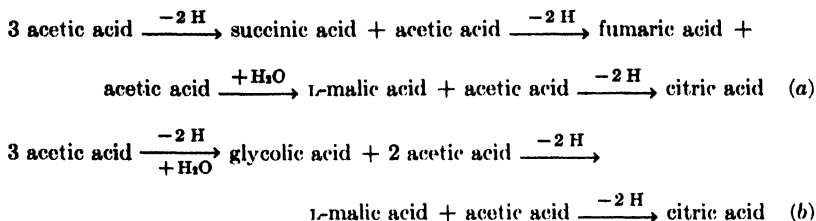
Taken together, the facts cited in the foregoing paragraphs afforded a strong clue to a possible route to citric acid, and it was Chrzęszcz and Tiukow (39) who took the next logical step, that of seeking for citric acid in acetate solutions subjected to the action of citric-acid-forming molds. They obtained the following results: From a solution of calcium acetate placed beneath the mycelium of *Penicillium citrogenum*, succinic and L-malic acids were obtained. Sodium acetate under similar conditions was converted to a mixture of L-malic, fumaric, and citric acids and in experiments with potassium acetate the products were succinic, L-malic, oxalic, and citric acids.

Later, Chrzęszcz *et al.* (40) studied the behavior of *P. johannioli* Zal., *P. citrogenum*, *P. citreo-nigrum* Dierckx, and *P. aurifluum* Biourge toward dilute solutions of alcohol in the presence of calcium carbonate. Mycelial "felts," grown previously for several days on a malt extract medium and then washed, were brought into contact with the alcohol medium. Under these conditions *P. citrogenum* produced acetate, fumarate, L-malate, and citrate. *P. johannioli* gave rise to formation of acetate, fumarate, glycolate, and citrate. *P. citreo-nigrum* afforded acetate, malate, and citrate and, from the cultures of *P. aurifluum*, acetate, malate, oxalate, and citrate were obtained.

Those molds which produced much acid from sugar also produced much acid from alcohol and those which formed little acid from sugar formed little acid from alcohol. All the molds formed some alcohol from sugar under anaerobic conditions.

#### E. REACTION SEQUENCES SUGGESTED BY DIFFERENT INVESTIGATORS

On all this evidence Chrzęszcz *et al.* were led to the conclusion that on sugar solutions the molds produced acetaldehyde or alcohol, which immediately underwent oxidation to acetic acid and from that stage there were two possible routes to citric acid, namely (a) and (b):



Later, however, Chrzyszcz *et al.* (41) expressed the view that sequence (a) was the more likely path to citric acid and that it was probable that glycolic acid behaved as the precursor of oxalic acid rather than as an intermediate in citrate formation.

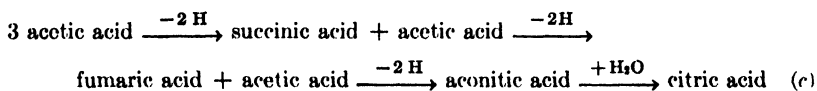
Experiments carried out at a later date by Yuill (167) are of interest in this connection. He found that *A. flavus* Brefeld gave rise to a very definite production of ethyl alcohol in a sucrose medium, in the presence of calcium carbonate, when the mycelium was somewhat water-logged but the conditions otherwise were aerobic. At the end of the period of incubation the metabolism solution was found to contain calcium citrate and soluble calcium salts, in addition to alcohol. These soluble salts were later (168) identified as malate, in considerable amount, together with succinate and fumarate. When this mold was caused to develop strictly upon the surface it gave rise to a heavily sporing mycelial mat, alcohol did not accumulate, and the solution was found to contain much calcium gluconate. It is of interest to note that Dammann, Rotini, and Nord (51) reported somewhat similar behavior on the part of a *Fusarium* species, which afforded high yields of alcohol from glucose and formed malic acid from alcohol. In the initial stages of the glucose fermentation the authors detected citric and oxalic acids. They concluded that alcohol is an intermediate in citrate production by *Fusaria*.

The views of Chrzyszcz and Tiukow found support in certain experiments of Suthers and Walker (136) and also in the experimental findings of Bernhauer and co-workers (10-12,14,17). Thus, addition of sodium sulfite to cultures of various strains of *A. niger* on glucose, under aerobic conditions, was shown by Bernhauer and Thelen to afford yields of acetaldehyde varying from traces to 20%. At the same time they observed that the fixation of the aldehyde had the effect of greatly depressing or stopping completely the

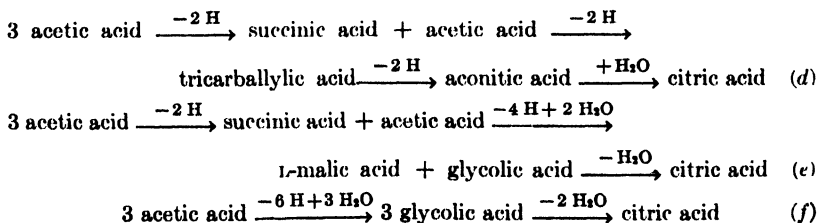
formation of citric and oxalic acids. Virtanen and Pulkki (145) also noted the inhibiting effect of sulfite on citrate formation.

In other experiments with ethyl alcohol as substrate in presence of sodium bicarbonate there were obtained, according to the strain of mold employed, yields of citric acid varying from traces to 25%, calculated on the alcohol assimilated. Bernhauer and Siebenäuger previously had reported the presence of small quantities of malic acid in sugar cultures of *A. niger*, and small quantities of the same acid, together with similar amounts of tartaric and oxalic acids, were now detected in the cultures on the alcohol medium. Traces of a reducing substance, thought to be a sugar, were also shown to be present in the latter cultures.

Butkevich (26) had already suggested that formation of citric acid might proceed by the steps:



Bernhauer *et al.* now showed that citric acid could be derived from aconitic, tricarballic, or glycolic acid when any one of these was used as sole source of carbon in an *A. niger* culture. Schoen (128) held that citric acid in mold cultures arises by the condensation of 3 molecules of glycolic acid, the latter being presumed to arise by oxidation of acetaldehyde. Bernhauer was of opinion that his own results justified consideration of the following additional schemes as possible pathways from acetate to citrate in mold cultures:



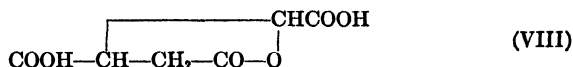
It was considered that in presence of *A. niger* there occurs in (d), between tricarballic and aconitic acids, an equilibrium comparable to the fumaric-malic equilibrium, which is catalyzed by fumarase. Reaction (d), if realized, would therefore constitute a strict parallel to the conversion of acetic acid to malic acid *via* succinic and fumaric acids.



Some years later Ciusa and Brüll (49) found that addition of glycolic and malic acids to cultures of *A. niger* on sucrose increased very greatly the yield of citric acid, and from this fact they concluded that the last stage in the synthesis of the latter acid consists in the condensation of 1 molecule of glycolic acid with 1 molecule of malic acid as in (e).

It will be obvious that still other hypothetical reactions could be drawn up to explain the formation of citrate from acetate, or from alcohol, under the experimental conditions mentioned in the above citations. For example, one might envisage a dehydrogenative condensation between 1 molecule of glycolic acid and 1 molecule of succinic acid to yield isocitric acid. The latter has been found in certain plants (100) and is converted into aconitic acid and thence into citric acid by the enzyme aconitase.

Another possible condensation would be that of glyoxylic acid with succinic acid to yield lactoisocitric acid (VIII), which, in turn, could readily be converted to citric acid. Succinic acid is known to



condense with aldehydes *in vitro* (56) to form  $\gamma$ -lactones related to isocitric acid, and glyoxylic acid has been detected in cultures of *A. niger* both on an acetate medium (38) and on a glucose medium (unpublished observation of the author). The possibility of the occurrence of this last condensation in animal tissues has been discussed by Krebs (88).

#### IV. Assimilation and Dissimilation of Organic Acids

##### A. ACTION OF *ASPERGILLUS NIGER* ON CITRIC ACID AND ON CITRATES

Although there is available a fair amount of evidence concerning the breakdown of citric acid by bacteria there have been relatively few studies of its degradation by molds. Challenger, Subramaniam, and Walker (38), employing one strain of *A. niger*, identified acetonedicarboxylic acid, acetone, malonic acid, glyoxylic acid, and traces of acetic acid among the products of citric acid breakdown and the action of this mold on these substances was, in turn, ascertained (Table I).

In order better to detect the presence of intermediates, the substrates shown in Table I were submitted to the action of the mold

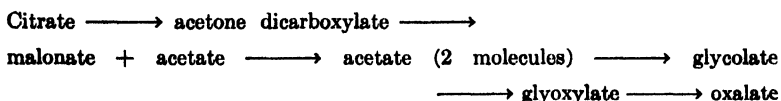
TABLE I  
SUBSTANCES FORMED IN CULTURES OF *A. niger*  
ON CITRIC ACID

Substrate	Substance produced	Derivative isolated and characterized
Ammonium citrate (20 g.) + citric acid (4 g.)	Acetonedicarboxylic acid	Diphenylhydrazone of mesoxalaldehyde (1.44 g.), m.p. 175-176°
Citric acid (45 g.)	Acetone (6 g.)	Isolated as such and converted to dibenzalacetone, m.p. 112°, and to the <i>p</i> -nitrophenylhydrazone, m.p. 148°
Citric acid (30 g.)	Malonic acid (3.58 g.)	Isolated as free acid and in part, as hydrogen formazyl, m.p. 119°, and as cinnamylidene-malonic acid, m.p. 207°
Citric acid (70 g.)	Acetic acid	Sodium salt (2.4 g.)
Citric acid (50 g.)	Glyoxylic acid	Aminoguanidine derivative (0.4 g.), m.p. 154°
Ammonium acetone dicarboxylate (20 g.)	Oxalic acid	Calcium salt (1.6 g.)
Malonic acid (20 g.)	Glyoxylic acid	Dixanthylhydrazone (0.5 g.), m.p. 147°
Malonic acid (25 g.)	Oxalic acid	Calcium salt (0.5 g.)
Calcium acetate (60 g.)	Glycolic acid (5.7 g.)	Isolated as free acid, m.p. 79-80°
Calcium acetate (20 g.)	Glyoxylic acid	Aminoguanidine derivative (1.2 g.), m.p. 154°
Calcium acetate (12 g.)	Oxalic acid	Calcium salt (< 1 g.)

in low concentrations, usually of the order of 1%, and the inorganic salts used in making up the media were the minimum quantities recommended by Molliard (104). Further, the mold was grown from spores sown on the media contained in large spherical flasks, the volumes of culture liquid being such that there was more liquid per unit of surface area than is usually the case in work with conical flasks. The products were trapped by adding suitable reagents to culture liquid withdrawn from the flasks, hence these products represent substances produced by enzymic processes proceeding normally, as distinct from those conditioned by the influence of "agents" added to the medium and remaining in it during the progress of events.

On the basis of these results the authors suggested tentatively that the decomposition of citric acid by *A. niger* follows the course

shown in Scheme II. Acetone was assumed to arise by decarboxylation of those molecules of acetonedicarboxylic acid which were not degraded to malonate and acetate. Evidence respecting the mode of degradation of citrate by *Pseudomonas pyocyaneum*, which produced a 67% yield of acetone (34), supports Scheme II.



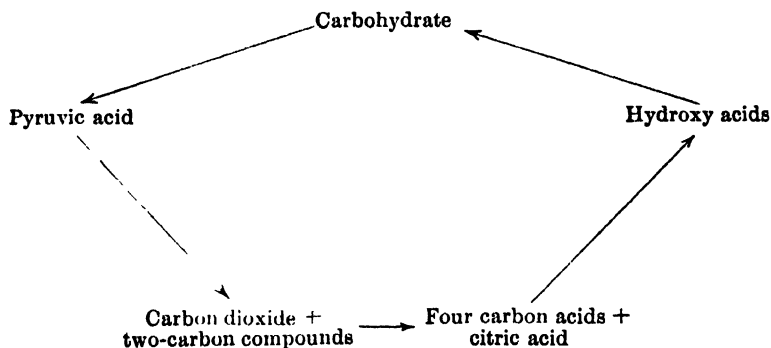
#### SCHEME II

*Behavior of Aspergillus niger in a "Starvation" Phase.* Scheme II has been criticized by Bennet-Clark (7) on the grounds that it does not really represent the fate of the citric acid produced and metabolized during the life cycle of an *A. niger* culture on glucose. The sequence of changes shown in Scheme II would involve the splitting off of at least 1 molecule of carbon dioxide for the disappearance of each equivalent of titratable acid during the course of the changes. Bennet-Clark and Le Touche studied the fate of citric, malic, glycolic, and glyoxylic acids when these severally were brought into contact with cultures of *A. niger* in a "starvation" phase and found that these acids were assimilated without any additional output of carbon dioxide. Presumably in these cases decarboxylation does not occur and Bennet-Clark considered that the added acid was probably reduced to an aldehyde, which was then utilized by the starving mold for synthesis of reserve carbohydrate.

Like Raistrick and Clark (121) and Bernhauer and Scheuer (12), Bennet-Clark did not obtain oxalic acid when glycolic acid was the substrate. Rapid disappearance of glycolic acid was attributed either to its conversion to glycolaldehyde or to glyoxal, followed by polymerization to carbohydrate, or to its conversion by a Thunberg reaction into some other acid such as malic acid, which then was presumed to be reduced to an aldehyde and polymerized to carbohydrate. Citric acid, it was suggested, might first be split hydrolytically to malic and glycolic acids, which would then undergo reduction' as already explained. Bennet-Clark was unable in the course of his experiments to secure direct evidence of the formation of these hypothetical aldehydes, but it is possible, nevertheless, to quote from other sources certain data which could be regarded as support for his view. The purely chemical reduction of the carboxyl

group to the aldehyde group is a laboratory process necessitating the use of a very vigorous reducing agent and established instances of such a transformation's being effected by biological means do not readily spring to mind. Kühnau (90), however, working with liver tissue, observed the conversion of  $\beta$ -hydroxybutyric acid to a mixture of acetoacetic acid and the aldehyde of  $\beta$ -hydroxybutyric acid and this, he stated, was the first example to be reported of the biological reduction of  $-\text{COOH}$  to  $-\text{CHO}$ . Assuming that reduction of this type can occur in the mold, the polymerization of the resulting aldehydic substance might be expected to proceed at a high rate and there would be little chance of detecting its presence (*cf.* Wirth and Nord, 160).

Bennet-Clark concluded that by the action of acid-forming molds on sugar the reactions of alcoholic fermentation first take place, yielding pyruvic acid, which then forms the starting point for the synthesis of the four-carbon dicarboxylic acids and citric acid, the complete cycle being that shown in Scheme III. He held Scheme



SCHEME III

III to be preferable to former hypotheses relating to citrate formation in molds, since none of these early proposals adequately explains the accumulation and disappearance of citric acid, which can be explained by Scheme III if it is assumed that the velocity of formation exceeds that of reconversion of the acid. The energy required for the postulated resynthesis of carbohydrate was presumed to be made available by oxidation of part of the acid or of some other substance.

As mentioned earlier, citric acid first accumulates in sugar cul-

tures and later disappears gradually. If such cultures are analyzed at regular intervals it will usually be found that the disappearance of citrate commences before the glucose is entirely utilized. That is to say, citrate is being metabolized in presence of the glycolytic products of the sugar. Now this was not the state of affairs either in the experiments of Challenger *et al.* or in those of Bennet-Clark for, in the former case, the mold was grown on citric acid or on one of its degradation products, and hence had to obtain the energy necessary for its synthetic processes by oxidation of a portion of the substrate, while in the latter case the single organic acids were fed to an already formed mycelial felt which had been brought into a "starvation" phase. The differences in the experimental conditions of the respective workers account for the fact that Challenger *et al.* were able to demonstrate the production of substances not detected either in the experiments of Bennet-Clark or in the earlier work of Raistrick and Clark and of Bernhauer and Scheuer. The formation of oxalic acid from glycolic acid affords such an example. Cases such as these illustrate well the difficulties in the way of reaching conclusions respecting reaction sequences when the experimental evidence has to be obtained under conditions which cannot be regarded as strictly physiological. Numerous similar cases could be quoted; for instance, there is the question of the fate of acetate in the yeast cell, investigated by Wieland *et al.* (131, 156-159; *cf.* also the conclusions of Kleinzeller, 77, on the same subject).

#### B. ACID METABOLISM IN RELATION TO RESERVE CARBOHYDRATE

Butkevich, who had at first adopted the view that citrate formation from acetate proceeds by way of succinate, fumarate, and aconitate, later formed a different opinion and, with Menshinskaja and Trofimova (29,30), stated that acetates are not converted to citrate by any of the reactions of dehydrogenation reviewed in an earlier section of this article. He now affirmed that acetates help the accumulation of citrate only insofar as they (acetates) exert a strong arresting action on consumption of citrate. Mycelia which enabled citrate to be produced from solutions of acetate were stated to contain more or less considerable quantities of a substance which reduced Fehling's solution, and it was suggested that citrate was

formed from this reserve carbohydrate and that oxalic acid was the chief product arising from the direct utilization of acetate. Butkevich and Osnizkaja (31) later extended this argument to the case of succinate formation from sodium acetate and claimed to have shown that because acetate "protected" succinate from the mold's attack, the succinate formed in cultures on acetate arises from materials stored in the mycelium. The evidence offered in the case of succinate formation was scanty and, to the writer, does not carry conviction.

As already indicated, the behavior of the mold varies considerably with the conditions imposed upon it and particularly is this so with respect to the hydrogen ion concentration of the medium. *A. niger* is unable to metabolize free acetic acid as sole source of energy and in experiments carried out in the writer's laboratory, sodium acetate served for the growth of *A. niger* only when present in solutions within the pH limits of 6.5 to 7.5. That being the case, it is impossible from experiments with salts of acetic acid to obtain exact information respecting the manner in which the mold deals with acetic acid produced during its growth on, for example, glucose or pyruvic acid.

This later view of Butkevich on the role of acetate was opposed by Bernhauer, who quoted experiments in which a mold placed on an acetate medium accumulated citric acid without decrease of mycelial weight, and he showed that the citrate formed in some cases had a weight greater than that of the mycelium. Such a counterargument cannot, however, be accepted as a complete refutation of that of Butkevich, since it might well be argued that the acetate had been progressively converted by the mold into reserve material to replace the reducing substance which, according to Butkevich, is initially present in the mycelium and undergoes conversion to citrate.

*Evidence from Use of Iodoacetate.* The writer with I. D. Chughtai, in hitherto unpublished work (42), has sought to obtain information on this question by employing iodoacetate in experiments with *A. niger* on an acetate medium. In conformity with the original observation of Lundsgaard (96) that carbohydrate resynthesis does not occur in yeast poisoned with iodoacetate, it was found that the latter at concentration 0.002 *M* completely prevented increase in the weights of young mycelial felts of *A. niger*, placed

on a new glucose medium containing the poison. In absence of the latter the weights increased threefold. In trials in which the mold was first developed fully on a sodium acetate medium, with subsequent replacement by fresh acetate solution, the presence of 0.002 *M* iodoacetate failed on the average to exercise any sensible effect either on the quantity of acetate consumed or on the weight of mycelium; moreover, the yields of the products, citrate and oxalate, were not markedly altered in the presence of the poison. In one experiment, two solutions, one of  $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$  (5.1 g. per 100 ml.) with 0.002 *M* iodoacetate, and the other of the same concentration without iodoacetate, were placed beneath the mycelial felts in two sets each of six flasks. These felts had previously been grown from spores during three days, and, at the time of replacement, the mean felt weight was  $0.530 \pm 0.043$  g. The media were analyzed on the third and fourth days after replacement and the felts were washed, dried at 60°C., and weighed at the end of the fourth day, when the weights were found to be: control flasks,  $0.630 \pm 0.05$  g., iodoacetate flasks,  $0.648 \pm 0.063$  g. The analytical figures for the products are shown in Table II.

TABLE II  
INFLUENCE OF IODOACETATE ON UTILIZATION  
OF ACETATE BY *A. niger*

Days	Acetate consumed, % of initial		Citric acid yield, % of acetate consumed		Oxalic acid yield, % of acetate consumed	
	Control	Iodoacetate	Control	Iodoacetate	Control	Iodoacetate
3	50.67	55.24	21.21	17.45	31.63	34.00
4	64.04	64.24	15.08	21.26	35.46	34.71

Further, it was observed that in cultures on sodium acetate the presence of citrate could be detected in the media at a very early stage, usually after 48 hours from the time of inoculation with spores, and before the mycelial felt was extensively developed. At such an early stage there could be little accumulation of reserve carbohydrate. While all these facts in no way disprove the claim that under certain experimental conditions production of citric acid from mycelial carbohydrate can take place, they do indicate very clearly that production of citrate from acetate by molds can proceed in a more direct manner not directly involving the carbohydrate reserve of the fungus.

*Multiple Factor Experiments.* It was decided to study acetate metabolism in *A. niger* by means of "multiple factor" or "factorial experiments" on the lines of the statistical techniques developed at Rothamsted Experiment Station by R. A. Fisher and colleagues. A detailed account of the method has been given by Brownlee (21). Studies on these lines of the products of *A. niger* in media of various compositions with acetate as the sole carbon source have been undertaken recently by D. J. D. Hockenhull and S. M. Shamsuz-Zoha, in association with the writer, and although the work is not yet complete the results obtained permit certain conclusions to be formulated. It was found that in certain media 90% of the sodium acetate supplied at about 3.5% level (calculated as free acetic acid) was consumed. The products were citrate and oxalate with a trace of glyoxylate. The highest yields, based on carbon consumed, were citrate 15%, oxalate 40%, and mycelium 20%. It appeared likely that oxalate was formed by direct oxidation of acetate, a confirmation of the verdict obtained from the experiments with iodoacetate discussed in previous paragraphs. More oxalate was produced when the initial pH value of the medium was 7.5 rather than 6.5, with ammonia rather than nitrate as nitrogen source and with high ammonia rather than low ammonia. Phosphate level had no effect. Citrate formation was not affected nearly so much by medium composition, although in general there was a slight advantage in using ammonia as nitrogen source. Certain conclusions, valid in general for this type of experimentation, were drawn. Considering first the production of oxalate, one may note that direct oxidation of acetate to oxalate changes three sets of variables: (1) a new acid equivalent per molecule is released, (2) a considerable oxidation is brought about, and (3) a large quantity of thermal energy is released. The production of oxalate is likely, therefore, to be stimulated by (1) a high pH value or any factors conducive to raising the pH value, *e.g.*, utilization of nitrate, and (2) any factors conducive to oxidation, *e.g.*, formation of reduced products of high thermal energy such as mycelial felt. In this respect it is highly probable that the stimulation of oxalate production by ammonium salts is due mainly to the thicker and heavier felt produced in their presence, and that the more reduced and acidogenic ammonium salt makes up in this way for its disadvantages as compared to nitrate. The latter (a) requires



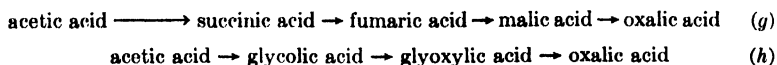
more reduction to form protein, and (b) releases base when it is thus reduced.

The conversion of acetate to citrate makes very little difference so far as the acid-base balance of the medium is concerned, in addition to which, citrate is much less oxidized and has a considerably greater thermal energy per carbon atom than has oxalate. Therefore, although the same factors operate as for oxalate formation, they do so to a much less extent, and a clear differential effect may be noted. Further, although oxalate production at the best levels of citrate is still decidedly high, there are hopes of eliminating the oxalic acid by alteration of other variables, such as the composition of the gas phase above the medium.

### C. SOURCES OF OXALIC ACID

Raistrick and Clark suggested that in glucose media oxalic acid is formed from acetic acid and from oxalacetic acid. They believed that the oxalate found when *A. niger* acts upon salts of succinic, fumaric, or malic acids is derived *via* oxalacetate. Very small yields of oxalate have been obtained from salts of acetic acid *via* glycolate and glyoxylate (38), but when oxalate is produced from acetate by mycological agency there is evidence to indicate that the major quantity is formed by way of succinate rather than through glycolate (12,29,32,41).

Nord and Vitucci (113) recently demonstrated the fact that in the cases of the wood-destroying mold *Merulius nivens* and related fungi oxalate can be formed from acetate by the two routes, (g) and (h):



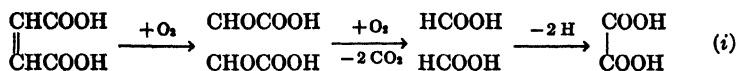
When route (g) was blocked by quinone, which inhibits succinic dehydrogenase, oxalate was still produced by route (h).

Allsopp (1) carried out a detailed investigation of the circumstances giving rise to oxalate production by growth of *A. niger* on the sodium salts of acetic, malic, and citric acids and on calcium gluconate, respectively, in a modified Czapek-Dox medium. No oxalate was found in corresponding cultures on the sodium salts of fumaric, maleic, lactic, pyruvic, and glycolic acids. When the free acid was used, growth was poor or absent, except in the cases of

succinic, fumaric, and citric acids. In the cases of succinic and citric acids, approximately the same amount of oxalic acid was obtained from the free acid as from the sodium salt. In experiments in which the free acids were fed to the mold in a "starvation" phase, the assimilation of gluconic acid was slow but oxalate finally appeared in the medium. It was calculated that 1 molecule of gluconic acid gave rise to 1 molecule of oxalic acid. The latter was not found in the media after assimilation of malic and pyruvic acids. From all his data he concluded that the oxalic acid which arises when cultures are reared on salts of many organic acids is a product of the carbohydrate reserves of the fungus and is trapped by the free base generated by the utilization of the organic anions. While there can be no doubt that Allsopp's experiments with the starved fungus demonstrated that oxalate formation can occur in the manner last mentioned, his claim that oxalate invariably arises in this way appears to be altogether too sweeping a generalization in face of the evidence, already cited, which other workers obtained in experiments conducted on different lines.

*Oxalic Acid from Sugars.* Allsopp also submitted sugars to the action of *A. niger* in a "starvation phase" and obtained oxalic acid from glucose, fructose, galactose, xylose, and arabinose. He arrived at the interesting conclusion that in such cases the oxalic acid arises by oxidation of the last two carbon atoms of a molecule with at least five carbon atoms in the chain. He suggested that formation of a keto acid is the first step and in the case of glucose supposed it to be fructuronic acid (D-2-ketogluconic acid) formed from gluconic acid produced directly from the sugar. The early stage at which oxalic acid arises in cultures of some strains of *A. niger* on sugars is in harmony with this view. There can be little doubt that oxalate formation in *A. niger* is distinct from citrate synthesis inasmuch as it does not depend upon a prior formation of the latter acid.

*Formic Acid as a Possible Source of Oxalic Acid.* In some experiments with *Aspergillus* species it was shown by Chrzęszcz and Zakomorny (41) that from salts of fumaric acid there could be formed in various cases formic, glyoxylic, oxalic, malic, and citric acids together with water and carbon dioxide. The production of oxalate from fumarate was thought to be effected by the stages shown in the following formulation (i):



According to this view fumaric acid by a purely chemical oxidation yields 2 molecules of glyoxylic acid which, by decarboxylation and simultaneous oxidation, are converted into 2 molecules of formic acid. Dehydrogenation of the latter could, it was stated, yield oxalate. The authors obtained a 77.6% yield of sodium oxalate by mycological conversion of sodium formate. Bernhauer and Slanina (16) also secured large yields of sodium oxalate from sodium formate and postulated the presence in the mold of an enzyme, formico-dehydrase, said to be analogous in its behavior to succinic oxidase. Butkevich, who had previously concluded that *A. niger* can oxidize sodium acetate directly to sodium oxalate, did not agree with this view of oxalate formation by dehydrogenation of formate. He cited (32) cases in which mycelial felts readily formed oxalate from acetate without change in weight but were quite unable to produce oxalate when kept on sodium formate solutions. In other experiments in which mycelial felts were placed on alkaline phosphate solutions there was considerable loss of mycelial weight and much oxalic acid accumulated. There is, however, no justification for the assumption that a particular mold which readily converts acetate to oxalate should be equally well able to convert formate to oxalate, for the strains differ very considerably in their respective degrees of ability to metabolize the salts of different acids. There is no doubt that Butkevich established his claim that oxalate can be formed from reserve material in the mycelium under certain specified conditions, namely, in presence of alkali, but this fact does not thereby prove that oxalate cannot arise also by the mechanisms suggested by Chrzaszcz and Zakomorny and by Bernhauer and Slanina.

## V. Formation of Gluconic Acid

### MÜLLER'S GLUCOSE OXIDASE AND ITS IDENTITY WITH NOTATIN

Optimum conditions for the laboratory preparation of gluconic acid by use of *Aspergillus niger* were determined by Bernhauer and the production of this acid on a larger scale was subsequently studied by a number of workers (66,101,102). High yields were

obtained when *Penicillium chrysogenum* was employed and the acid has been obtained both in pan culture and in cultures in drums under increased air pressure.

Müller (107) obtained enzyme preparations from the expressed juices of *A. niger* and of *P. glaucum* by precipitation with 90% alcohol or with alcohol-ether; these preparations oxidized glucose to D-gluconic acid. He termed the enzyme "glucose oxidase" and after further study of its behavior (108) he concluded that two enzymes were present, one acting directly with oxygen and the other reacting only with certain other hydrogen acceptors, for example, with 2,6-dichlorophenol-indophenol.

Franke and Lorenz (60) also separated from *A. niger* and from *P. glaucum* an enzyme preparation which behaved as a glucose dehydrogenase, oxidizing glucose to gluconic acid and utilizing molecular oxygen or methylene blue as hydrogen acceptor. Further purification of this glucose-oxidizing enzyme was carried out by Franke and Deffner (59), who established the fact that the prosthetic group is a flavin and that under its influence and in aerobic conditions the oxidation of glucose proceeds by reduction of molecular oxygen to hydrogen peroxide. They stated that the enzyme is cyanide-insensitive.

The history of the glucose oxidase of molds really begins with the observation of Raciborski (120) that in a glucose medium *A. niger* excretes an iodide-oxidizing system and that both phenol-oxidases and peroxidases are absent. Hence, he postulated the existence of a specific iodide oxidase. Korczewski (83) detected hydrogen peroxide in the medium but concluded from its low concentration that it could not be responsible for the oxidation of iodide. Korczewski thought that the medium contained an oxygenase similar to the type of enzyme postulated by Bach and Chodat. This was supposed to form with molecular oxygen a superoxide, which effected the oxidation of iodide. The question was reinvestigated by Pearce (117), who discovered that the oxidation of potassium iodide was due solely to hydrogen peroxide formed in the primary oxidation of glucose catalyzed by Müller's glucose-oxidizing enzyme. Pearce also showed that the iodine so liberated could then compete with oxygen as a hydrogen acceptor. He pointed out that Müller's enzyme shares with other oxidizing systems the property of promoting secondary oxidation reactions. The addition

of alcohol and catalase to such systems doubles their oxygen uptake. In this reaction, as was demonstrated by Keilin and Hartree (74), the oxidation of alcohol to aldehyde by hydrogen peroxide formed in the primary oxidation reaction is catalyzed by a small concentration of catalase. An increase in oxygen uptake by the media in Pearce's experiments was also observed on addition of acetaldehyde, which was oxidized to acetic acid. This last-mentioned secondary oxidation reaction like the oxidation of iodide is, on the contrary, inhibited by catalase. Pearce also found, in conformity with the experience of others, that Müller's enzyme is not inhibited by potassium cyanide, sodium azide, hydroxylamine, or sodium pyrophosphate, all of which appeared to accelerate the oxygen uptake of the system. On the other hand, sodium fluoride and sodium acid sulfite considerably inhibited activity of the enzyme.

According to Knobloch and Meyer (80) the optimum pH value for the activity of Müller's enzyme is 4.7 to 6.0. These workers reported that *A. niger* converts mannose to mannonic acid and galactose to galactonic acid, presumably by the agency of the same enzyme. The activity was stated to fall off in the order glucose > galactose > mannose. Later, Keilin and Hartree (75) stated that galactose is not attacked, the result observed by Knobloch and Meyer being due probably to glucose as impurity.

Müller's enzyme can utilize not only oxygen but also iodine, quinone, *o*-chlorophenol-indophenol, 2,6-dichlorophenol-indophenol, toluylene blue, and thionine as hydrogen acceptors.

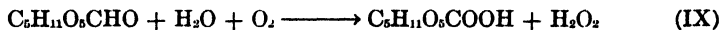
Harrison (65) discovered that liver extracts contain a dehydrogenase capable of oxidizing glucose to D-gluconic acid. This enzyme, however, unlike that from *A. niger*, could not utilize oxygen directly as hydrogen acceptor but, *in vitro*, readily worked in conjunction with methylene blue.

Ogura (116) described the isolation from *A. oryzae* of an enzyme which effected the oxidation of glucose to gluconic acid at a pH optimum of 7.8 and which, in contrast to Müller's enzyme, could not act directly with oxygen. It could function, however, by using 2,6-dichlorophenol-indophenol as hydrogen acceptor.

In later work, Franke (58) criticized the conclusion of Müller that there are two enzymes and the conclusion of Ogura that the enzyme of *A. oryzae* is different from Müller's enzyme. He considered the glucose oxidases from *A. niger*, *P. glaucum*, and *A.*

*oryzae* to be virtually identical and to consist of a single aerobic oxidase. He attributed the findings of the other workers to the low purity of their preparations.

The question of the nature of Müller's enzyme has now been settled in a most convincing manner by Keilin and Hartree (75) following the very thorough studies made by Short and colleagues (48) at Nottingham and by Van Bruggen *et al.* (122) in America. The Nottingham team effected the almost complete purification of glucose oxidase from culture media of *P. notatum* and demonstrated its identity with the antibiotic named "notatin." The same substance has also been designated "penatin" (*cf.* Kocholaty, 82). It has been found to occur also in the mold *P. resticulosum*. The antibacterial activity *in vitro* is very high and the substance completely inhibits the growth of *Staphylococcus aureus* at dilutions as great as 1 in 1,000,000,000, this effect being due to hydrogen peroxide formed in accordance with the reaction (IX):



The American workers described the same substance under the name "penicillin B" and their experiments suggested that the prosthetic group of the enzyme was the alloxazine adenine dinucleotide, a suggestion confirmed by Keilin and Hartree, who lately have definitely established this fact by removing the prosthetic group from D-amino acid oxidase and regenerating the activity of the latter by addition of boiled notatin. They worked up the enzyme to a state of extreme purity when it had  $\text{Q}_{\text{O}_2} = 130,000$  (Franke had found  $\text{Q}_{\text{O}_2} = 8000$  at  $30^\circ$  in oxygen) and molecular weight = 152,000, indicating two prosthetic nuclei.

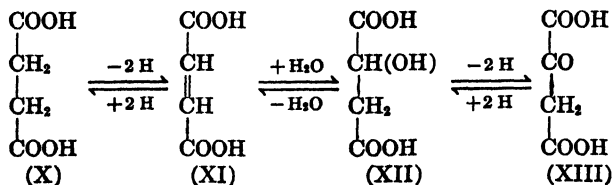
The highly purified enzyme showed pronounced specificity for glucose, though mannose, xylose, 6-methylglucose, and 2,6-dimethylglucose were oxidized at about 1% the rate of glucose. No other sugar was attacked. Finally, Keilin and Hartree state that the respiration of *P. notatin* is cyanide-sensitive, but the oxidation of glucose by the mycelium is not inhibited by cyanide and therefore the cytochrome system is not involved in the intracellular oxidation of glucose to gluconic acid. The last observation was also made by Bose and reported in a prior publication (20).

## VI. Implications of the Discovery that Carbon Dioxide Is Fixed in Molds

### A. REVISION OF THE THEORY THAT FORMATION OF CITRIC ACID DEPENDS ON PRIOR ZYMESIS

The reaction sequence of Chrzaszcz and Tiukow leading to citric acid hinged on the possibility of the occurrence in the mold of the Thunberg dehydrogenation of acetic acid to succinic acid and, although this had not been unquestionably demonstrated enzymically, there was ample indirect evidence in favor of its occurrence in living cells (81,90,106,119,154,161). Proof of the ability of a microorganism to effect this synthesis was finally provided by Slade and Werkman (130) who, using  $^{13}\text{C}$  as a tracer, showed that cell suspensions of *Aerobacter indologenes* can effect the dehydrogenation of two molecules of acetic acid with a 13% yield of succinic acid. Further experiments (72) with cell-free enzyme preparations from *Escherichia coli* substantiated this result.

This demonstration makes easier of acceptance the idea of the other dehydrogenations envisaged in the Chrzaszcz-Tiukow hypothesis and in the similar schemes of Bernhauer. It is not clear, however, why these last-mentioned workers, when formulating possible reaction sequences, entirely overlooked the original idea of Raistrick and Clark that the immediate precursors of citric acid are acetic acid and oxalacetic acid, because once succinic acid has been formed in the mold it can give rise to oxalacetic acid by way of the reactions involved in Szent-Györgyi's reversible enzymic systems (X to XIII):



If this view be held it is unnecessary to assume a dehydrogenation between acetic acid and a four-carbon dicarboxylic acid, for the former could be supposed to participate with oxalacetic acid in an aldol condensation leading directly to citric acid. It is generally supposed (88) that condensation of acetate with oxalacetate can-

not take place in animal tissue because of the very low reactivity of acetate in the system, but it does not thereby follow that the same state of affairs prevails in the mold.

*Evidence That Citrate Formation Is Not Dependent on the Phosphorylating Mechanisms of the Zymase System.* The possibility that any hypothesis of citric acid formation, based on the assumption that acetaldehyde is an essential precursor, might prove to be ill-founded was foreseen by Virtanen and Pulkki (145) before the views of Chrzęszcz and Tiukow were developed, for the former workers drew attention to a claim of Butkevich to have obtained citric acid in 85.5% yield from sugar and they pointed out that so high a yield would have been impossible had the formation of the acid depended on the supply of acetic acid derived *via* a prior process of alcoholic fermentation. Later, certain experiments undertaken by Johnson (70) and confirmed by Johnson, Knight, and Walker (71) clearly showed that citrate-forming *aspergilli* and *penicillia* certainly do possess a pathway (or pathways) from sugar to citrate, which is independent of the Embden-Meyerhof processes of alcohol formation. In these experiments it was found that certain strains of *A. niger* and of *Penicillium* species could give normal yields of citric acid in the presence of 0.002 *M* iodoacetate, which inhibited the formation of alcohol by these molds under anaerobic conditions.

*Evidence from Yield of Product.* At about the same time Butkevich and Gajevskaja (28), Wells, Moyer, and May (153), and Clutterbuck (45) claimed to have obtained yields of citric acid greater than the maximum yields (106.7% by weight from sucrose and 71.1% from glucose) which could be obtained were it to be derived from acetic acid which, in turn, had arisen *via* alcoholic fermentation in the mold. Wells *et al.* also pointed out that if citric acid is derived from sugar *via* alcohol, the citric acid to carbon dioxide weight ratio should not exceed 1.45/1. They found this theoretical ratio to be greatly exceeded. Further evidence substantiating the results of Wells came from quantitative estimations made by Tomlinson (141), who studied anaerobic metabolism in relation to citrate formation in *P. divaricatum* Thom and *P. sanguifluus* (Sopp) Biourge.

In view of all these findings Walker *et al.* (70,71) were led to suggest that formation of citric acid from glucose may involve



initial breakdown of the sugar molecule on the lines indicated by Scheffer and Kluyver (78,126), in seeking to explain the behavior of certain bacteria toward glucose. According to this view the sugar was presumed to undergo scission to ethylene glycol and the dialdehyde of tartaric acid, from which, in turn, there would arise acetic acid and succinic acid, respectively. Alternatively, acetic and oxalacetic acids could be formed (162). Either of these pairs of acids might then be expected to yield citric acid by one or other of the processes already discussed. A somewhat similar suggestion that scission of glucose into a two-carbon molecule and a four-carbon molecule might precede formation of citric acid came at about this time from Gudlet, Kirsanova, and Makarova (64).

*Fixation of Carbon Dioxide by Aspergillus niger.* In 1941, however, following Wood and Werkman's (163) discovery that propionic acid bacteria bind carbon dioxide in pyruvic acid, there came proof of the fixation of carbon dioxide in the synthesis of fumaric acid in *Rhizopus nigricans* and of citric acid in *A. niger*. This was demonstrated by Foster, Carson, Ruben, and Kamen (57) in tracer experiments and at once served to explain the results obtained by Wells *et al.* and by Tomlinson.

This new information necessitated reconsideration of the Chrzęszcz-Tiukow hypothesis, for, if the reactions of alcoholic fermentation proceed in the mold to the stage of pyruvic acid and this is followed by fixation of carbon dioxide to yield oxalacetic acid, the synthesis of citric acid can be presumed to follow by way of the hypothetical oxalocitraconic acid (Wood's pyruvofumaric acid) or more probably *via* a condensation of oxalacetic acid with acetic acid or with a two-carbon substance related to the latter (97,99,152), for example,  $O=C(OR)-CH_3$ , where R may be, for instance, a phosphate radical. While the original Chrzęszcz-Tiukow hypothesis allowed at the most a yield of 128 g. of citric acid from 180 g. of glucose, the maximum yield from that weight of sugar would be 192 g. were the reactions to proceed according to this amplified scheme, which thus serves to account for the high yields obtained by Butkevich, by Clutterbuck, and by Wells *et al.*

It is thus possible to view citric acid formation in molds in relation to that of the tricarboxylic acids of the Krebs cycle and the further question arises whether the full cycle occurs in the mold or whether the reactions terminate in the production of citric acid.

## B. REACTION SWITCHING AND THE TRAPPING OF PYRUVIC ACID

It is convenient at this stage to anticipate certain results which it is hoped will be published in full elsewhere. These relate to experiments undertaken with the object of identifying intermediates arising during the operation of the acid-forming processes in the mold. Many attempts have been made to detect transitory products in cultures developed in the absence of fixation agents but, apart from the identification of malic acid in very small quantities and, in a single case, of a trace of tartaric acid, no substance (other than gluconic acid) which could be regarded as a precursor of citric acid has hitherto been isolated. Malic acid was detected by Bernhauer *et al.* (10) in the culture media of 28 strains of *A. niger* which were producing citric acid from sucrose; in the Manchester laboratories it has been detected on numerous occasions in glucose media about the time when citric acid first makes its appearance. The quantity present at any one time is, however, very small. Hence, it must be concluded that in such circumstances the mycological formation of malic acid bears a definite relationship to that of citric acid.

Bernhauer *et al.* trapped acetaldehyde (13,17) in sugar cultures of *A. niger* by use of sodium sulfite, but the presence of the latter either strongly suppressed citrate formation or stopped it altogether, an effect which Bernhauer and Schön believed due to development of alkalinity in the medium. Small quantities of both pyruvic acid and dimethylpyruvic acid have also been trapped by the use of sulfite under specially defined conditions (67) in which formation of citrate was not entirely suppressed.

In the past the writer has tried repeatedly, but hitherto always unsuccessfully, to establish the formation of pyruvic acid in mold cultures on glucose in more normal conditions than can exist in the presence of sulfite. For example, the action of basic magnesium phosphate, as used by Neuberg and Kobel to demonstrate the production of pyruvic acid from sugar by yeast at pH 8 to 5, was tested, but in mold cultures no special effects were observed following the use of this agent.

*Effects of Sodium Arsenite.* Recently, it was decided to study the effects of arsenite, since this substance is known to exert a protective influence on certain  $\alpha$ -keto acids by inhibiting oxidative

decarboxylation, cf. Krebs, and Krebs and Johnson (87,89). Chughtai (42) finds that in a glucose-inorganic salts medium, sown with spores of *A. niger*, arsenite causes no restriction of growth at concentrations below 0.004 *M*. In cultures developed from spores in the presence of arsenite at this concentration, sugar utilization and acid production are both accelerated as shown by comparison with "controls" developed in the absence of this agent. Using pre-formed mycelial felts it was established that sugar consumption in a given time could be doubled by addition of arsenite (0.004 *M*) and in the same time production of citric acid could be increased to 3.5 times that formed in the control. The total acid produced in the presence of arsenite was also of the same order, being 3.5 times that produced in the control. Further evidence of enhanced metabolic activity in the presence of arsenite was afforded by the weights of the mycelial felts which, developed respectively in the presence and in the absence of the fixative agent, were in the ratio 1.4/1. Finally, it was found that 0.004 *M* arsenite had reduced very greatly the carbon dioxide loss, which amounted only to about one-seventh of the loss suffered in the control.

It is thus clear that arsenite at a defined concentration can switch the mold's metabolic activities further in the direction of citric acid formation.

*Detection of Pyruvic Acid.* It was found that the stabilizing effect of arsenite was such that in its presence pyruvic acid could accumulate in very small quantities in glucose media in which citric acid was being formed. The former acid was isolated as the 2,4-dinitrophenylhydrazone from cultures containing arsenite at concentration 0.01 *M*. The latter at a concentration of 0.02 *M* allowed more pyruvic acid to accumulate and depressed greatly the formation of citric acid. In these experiments oxalacetic and  $\alpha$ -ketoglutaric acids were sought for in the media but they were not detected.

*Effects of Sodium Cyanide.* Although this agent has profound effects on many biological systems, inhibiting, for instance, the activities of catalase, peroxidase, tyrosinase, and cytochrome *c*, it does not seem to have much direct action on certain dehydrogenases, e.g., notatin, and in fact cyanide can be used as a source of nitrogen by *A. niger* in a condition of nitrogen starvation and its utilization causes increase of mycelial weight (68,69). Dammann, Rotini, and

Nord (52) have shown that under appropriate conditions certain physiological activities of the mold *Fusarium graminearum* Schwabe are stimulated by cyanide, which increases the weight of felt and causes fermentation of pentoses, particularly of L-arabinose, to proceed at a higher level of activity accompanied by a greatly increased yield of alcohol.

Many years ago it was observed in these laboratories (79) that cyanide (0.002 *M*) markedly activated certain processes in a *Penicillium* species on a glucose medium, with the result that in a given time sugar consumption and formation of citric acid were nearly doubled and there was also considerable increase in the yield of gluconic acid. Gale and Stephenson (62) found cyanide (0.06 *M*) to be the only effective fixative for oxalacetic acid produced by the action of the malic dehydrogenase of *Escherichia coli*, since the product is obtained as the cyanhydrin and, after liberation of the free oxalacetic acid, the latter may be isolated in the form of its 2,4-dinitrophenylhydrazone. On the basis of these facts it seemed feasible that the use of cyanide might render it possible to trap ketonic intermediates in citric acid formation and, in particular, oxalacetic acid was sought in glucose cultures of *A. niger* to which cyanide had been added. In this work A. M. Nield and I. D. Chughtai, using preformed felts, observed that cyanide exercised effects very similar to those produced by arsenite. Optimum effects were noted at 0.02 *M* cyanide, at which concentration the weight of the mycelium increased by about 40 to 50% compared to that of the control. The respective rates of sugar dissimilation and acid production rose to a very marked extent and there was a reduction in the output of carbon dioxide. In some experiments this sparing of carbon dioxide amounted to as much as 23%. Attempts to trap intermediates were, however, unsuccessful.

Enhancement of activity due to cyanide, with resultant increase in the yields of the acidic products, is not due solely to the fact that this agent causes appreciable increase in the size of the mycelium. Cyanide exercises an inhibitory effect on the respiration of *A. niger* (98) and, in so doing, it displaces the balance of the organism's activities in the direction of fermentation. In such a case the final result, namely, an increased production of citric acid, is further evidence for the view that the synthesis of the latter has, as its starting point, a product or products of zymasis. In support of

the same thesis still further evidence, based on other reasoning, was obtained by use of adenosine triphosphate.

*Effects of Adenosine triphosphate.* According to the role this substance (ATP) has in the operations of the zymase system its effect on *A. niger* should be to intensify the processes leading from sugar to phosphoenolpyruvic acid. Apart from the fact that ATP functions in phosphorylating degradation at different stages on the path from glucose to pyruvic acid, this cofactor is believed (144) greatly to assist the fixation of carbon dioxide in oxalacetate and, according to Ochoa (114,115), ATP also brings about an inhibition of the enzymic decarboxylation of oxalacetic acid at pH 5.0 and in presence of 0.001 *M* manganese chloride. On these grounds, on the supposition that citrate formation in glucose media depends on prior formation of pyruvate, followed by fixation of carbon dioxide, adenosine triphosphate should increase the rate of formation of citrate. That such actually is the case was shown by K. Ramachandran and I. D. Chughtai in an experiment in which ATP was employed at a concentration of 300  $\gamma$  per 100 milliliters in a glucose medium introduced beneath a ready-formed mycelium. At the end of 48 hours sugar consumption was unaffected by the ATP, but formation of citric acid was 50% higher in its presence as compared to the amount of this acid formed in the control.

### C. ISOLATION OF ACETIC ACID IN CITRATE FORMATION

*Isolation of Acetic Acid from Mycelium.* While it has not proved possible to detect acetic acid in the metabolism solutions under young mycelial felts of *A. niger*, Pearce and Chughtai (43) have noted its presence in the mycelium at the time when citric acid production is becoming well marked. The felts from three strains gave only very small yields of acetic acid when disintegrated and treated with steam, but a fourth strain proved a better source of the acid and in two experiments appreciable yields were obtained. The acid was characterized as its silver salt and also as the *p*-bromophenacyl ester. The yields of acid given by different strains bore a rough relationship to the citrate-forming capabilities of the strains. In all these experiments, at the times when acetic acid was found to be present in the mycelia, the culture media gave a decided color reaction with naphthoresorcinol and concentrated hydrochloric acid, the color being similar to that given by gly-

oxylic acid. However, attempts to prepare a derivative of the latter were not successful, owing perhaps to the presence of much glucose. The coloring matter given by glyoxylic acid in this test is soluble in ether, but it is very difficult, if not impossible, to distinguish it from the purple coloring matter given in the same test (Tollens) by glycuronic acid, for this also dissolves in ether. The possibility that glycuronic acid was present in the metabolism solutions cannot, therefore, be excluded. Glyoxylic acid, however, can be detected readily in the culture liquid when *A. niger* grows on a sodium acetate medium, so the occurrence of glyoxylic acid in glucose cultures of *A. niger* is understandable now that acetic acid has been isolated from the mycelium.

The acetic acid found in the mold mycelium could have arisen from pyruvic acid in one of several possible ways: (a) by decarboxylation to acetaldehyde, for *A. niger* secretes carboxylase (17, 109, 148), and subsequent dehydrogenation of acetaldehyde hydrate; (b) by oxidative decarboxylation (5, 6, 135), and (c) by the action of pyruvic dehydrogenase on pyruvic acid phosphate (92, 142, 143). With respect to c, in which phosphate would be involved, the acetic acid would appear as acetyl phosphate and might condense as such with the oxalacetic acid, without the need first to be decomposed to free acetic acid (cf. 88, 97).

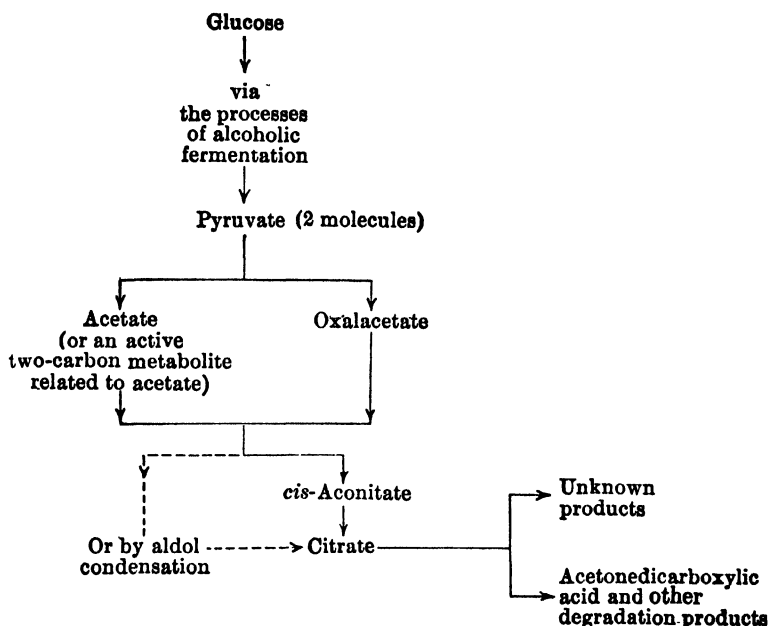
#### D. CONCLUSIONS WITH RESPECT TO FORMATION OF CITRIC ACID

*Under Normal Conditions.* Production of acids occurs by series of reactions which follow more than one pathway. One of these proceeds by way of gluconic acid, but what is presumed to be the main route follows the stages of alcoholic fermentation up to the point at which pyruvic acid arises. It has not been conclusively shown that this pyruvic acid is produced by the operation of the phosphorylating mechanisms of the Embden-Meyerhof scheme, but the fact that carbon dioxide is fixed supports the view that phosphorylated metabolites are involved in some at least of the acid-forming processes. Possibly in *A. niger*, as in the *Fusarium* species studied by Nord *et al.* (110, 111), there can occur a phase of phosphorus-free fermentation. The matter certainly merits inquiry since a degradation of glucose by processes other than the Meyerhof sequence has been demonstrated in *A. tamarii* Kita by Gould (63)

who, inhibiting phosphorylation and organic phosphate breakdown by sodium fluoride and by iodoacetate, was yet able to obtain kojic acid from the cultures. Mann (98) made important contributions to our knowledge of phosphorus metabolism in *A. niger*, but his results do not throw light on the processes of alcoholic fermentation in the mold. He showed, however, that the phosphorus metabolism is strictly aerobic in character. In this respect the mold differs from yeast, which can utilize phosphate in the presence or in the absence of oxygen. Mann also found that an intimate relationship exists between the phosphorus metabolism and the respiration of *A. niger*, because cyanide (0.001 *M*), azide (0.001 *M*), iodoacetate (0.001 *M*), and fluoride (0.005 *M*) all strongly inhibited the mold's respiration and also brought to a standstill its phosphorus metabolism. He observed that by increasing the inorganic phosphate in the medium he could enhance the mold's content of aneurin, of riboflavin, and of nicotinic acid, and he also noted that the glucose oxidase activity of a mycelium grown on a deficient quantity of phosphate (0.02% potassium monohydrogen phosphate), was four times as great as that of a mycelium grown on ten times that quantity of the salt. There would seem to be a connection between this fact and the writer's observation that gluconic acid formation in *A. niger* can be stimulated by iodoacetate at concentrations between 0.001 *M* and 0.002 *M*.

Now, citrate accumulates in cultures of citrate-forming molds, whereas in animal tissues in which it is formed there is no accumulation. Moreover, experiments undertaken to identify the products of the mycological degradation of citrate have indicated that this proceeds to some extent, at least, by way of acetone dicarboxylate (38) and the conclusions of Deffner (53), who believed oxalacetic acid to be the primary breakdown product of citrate under the action of bacteria, constitute no criticism of the former view, because Deffner's experiments were performed anaerobically. Hitherto, the formation of  $\alpha$ -ketoglutarate during the mycological breakdown of citrate has not been reported. These facts strongly support the assumption that in molds the complete tricarboxylic acid cycle does not occur or, if it does occur, the extent of its occurrence is such as to account for only a small part of the citrate metabolized. It may be the case that isocitrate dehydrogenase is absent from the mold or, if present, that its activity is weak. If the latter is the case this

would constitute a parallel to that of carboxylase activity in certain species of *Fusarium*, in which the low activity of this enzyme allows pyruvic acid to accumulate (112). The same is true of certain species of *Candida* (unpublished observation of K. Ramachandran in this laboratory). When comparing the fate of citrate in the mold to its fate in the animal body it must also be borne in mind that conclusions reached from consideration of relative rates of enzyme reactions in one or more types of animal tissue must not be assumed



SCHEME IV

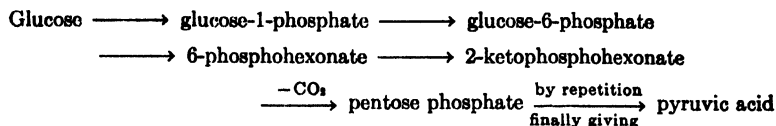
necessarily to be valid for systems in the mold, where pH values, concentrations of metabolites, and temperatures are different from those in the animal body. Furthermore, Ochoa (114) and Ochoa and Weisz-Tabori (115) have shown that by the operation of a particular enzyme system containing a specific carboxylase, which occurs in pig's heart, together with reduced triphosphopyridine nucleotide (TPN), isocitric dehydrogenase, and manganese ions, a reversal of a part of the tricarboxylic acid cycle can be effected,



whereby  $\alpha$ -ketoglutarate is converted to isocitrate by carbon dioxide fixation, and a further shift in the equilibrium of reaction occurs in the presence of aconitase. If the latter is present, over 90% of the L-isocitric acid formed is converted (at 37°) to *cis*-aconitic and citric acids. The simultaneous presence of a hexose monophosphate dehydrogenase system, which would re-reduce the oxidized TPN as it is formed, would further serve to maintain the continuance of these actions in reverse. Should such systems be present in the mold, a point on which we have as yet no information, they could be held responsible for the shunting of the reaction mechanisms along the side-track leading to citrate and its degradation products. All these ideas, however, have as yet no basis in experimental data, and must be regarded merely as possible hypotheses on which to frame further inquiries with respect to the fate of citrate in the mold.

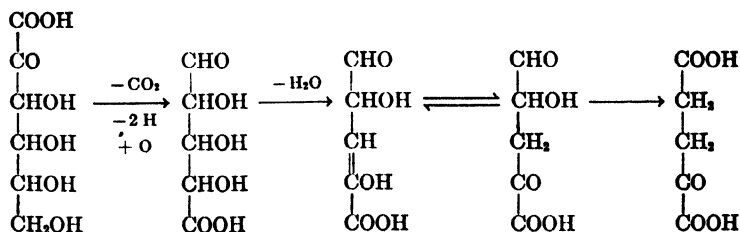
The whole of the matters discussed in the previous paragraphs of this section can be diagrammatically summarized as Scheme IV.

*In Presence of Iodoacetate.* The iodoacetate-resistant respiration of *A. niger* cannot involve phosphoglyceraldehyde dehydrogenase since this enzyme is sensitive to low concentrations of the poison. Now, glucose oxidase is resistant to iodoacetate, so if the formation of citrate from glucose in presence of iodoacetate proceeds not only through gluconic acid but also by some other sequence of changes, which involves glycolysis intermediates, the latter must be shunted before the triose phosphate stage is reached. Warburg and Christian (149) detected in animal tissues a direct oxidation of glucose-6-phosphate to phosphogluconic acid (6-phosphohexonic acid) by a system catalyzed by coenzyme II (TPN). Further oxidation by the same enzyme in the presence of proteins obtained from yeast gave a substance showing the reactions of a pentose. Further work on this topic by Lipmann (91) and by Dickens (54), who studied this aerobic dissimilation of phosphogluconic acid by yeast or by yeast preparations, led to a phospho-ketogluconic acid and a phosphopentonic acid. These results indicate that this direct oxidation of hexose phosphate involves successive oxidations and decarboxylations, which should eventually end in the production of pyruvic acid according to Scheme V. To what extent sugar oxidation in different types of tissue may be represented by this particular phase sequence is not yet known, but it would appear not unreasonable to suggest that these reactions



SCHEME V

might represent an alternative pathway in molds, and it is known to be relatively insensible to poisons such as iodoacetate (132). It remains to consider the production of citrate from D-gluconic acid formed by the action of glucose oxidase. We have as yet no evidence concerning the stages of this transformation; possibly it consists of a phosphate-free version of the sequences studied by Warburg, by Lipmann, and by Dickens. There is, however, another way of regarding the matter which, while it is no more than "paper-chemistry," will serve to suggest a possible line of attack on the problem. From glucose certain species of *Pseudomonas* and *Acetobacter* produce 2-ketogluconic acid. Lockwood and Stodola (95) have established that *Pseudomonas fluorescens*, if allowed to act upon glucose until the medium no longer shows a reducing action, gives rise to  $\alpha$ -ketoglutaric acid by way of 2-ketogluconic acid. It will be recollected that Tomlinson believed the formation of the last-mentioned acid to be one of the early stages in the production of citrate from glucose by *A. niger*. The mechanisms by which 2-ketogluconate could be transformed into  $\alpha$ -ketoglutarate could be formulated hypothetically as in Scheme VI.



SCHEME VI

From  $\alpha$ -ketoglutaric acid the formation of citric acid might be presumed to occur by one or other of two possible pathways: (a) by oxidative decarboxylation to succinic acid and thence along the

tricarboxylic acid cycle through oxalacetic acid, or (b) through isocitric acid by the tricarboxylic acid cycle acting in reverse, a possibility to which reference has already been made.

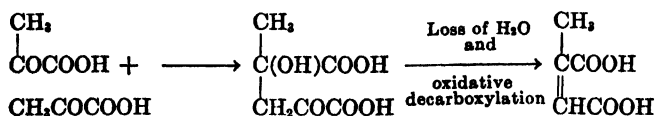
#### E. FORMATION FROM GLUCOSE OF FOUR-CARBON DICARBOXYLIC ACIDS, OF LACTIC ACID, AND OF ITACONIC ACID

In molds in which the enzyme necessary for the production of citrate from oxalacetate and a two-carbon metabolite, usually is not present, as is the case in the genus *Mucor*, it must happen that the enzymes of the Szent-Györgyi cycle come into play and from oxalacetic acid produce the other four-carbon acids of this cycle. The proportions of these acids found in the medium will probably be regulated by the oxidation-reduction potential set up in the system which gives rise to them.

The small quantities of tartaric acid sometimes detected in the cultures no doubt arise by addition of water to enoloxalacetic acid (123), though their formation by addition of the elements of hydrogen peroxide to the double bond of fumaric acid may be considered an alternative possibility (*cf.* Wieland, 155).

Lactic acid, which is sometimes encountered in sugar cultures of certain species of *Rhizopus*, is derived presumably by reduction or by dismutation of pyruvic acid.

Itaconic acid has been reported as occurring in sugar cultures of one or two *Aspergillus* species (35,76) and the factors which affect its production by *A. terreus* have been studied by Lockwood and Reeves (94) and by Lockwood and Nelson (93). While it is possible that itaconic acid arises by decarboxylation of *cis*-aconitic acid, the structure of the latter is not such as to indicate a liability to lose carbon dioxide and, in the writer's opinion, a more likely mode of formation of itaconic acid would be from pyruvic acid, as shown in Scheme VII.



SCHEME VII

Recently Yuill (169) has reported the formation of both itaconic acid and kojic acid in the same sucrose culture of a species of

*Aspergillus* with yellow to ochraceous-colored conidia. The ratio in which the two compounds appeared could be varied by varying the experimental conditions. The formation of these two substances in one culture poses the questions whether two separate processes of sugar dissimilation, one involving phosphorylation and the other being phosphate-free, are necessary for their respective modes of production, or whether the compounds arise by the branching of one main chain of reactions.

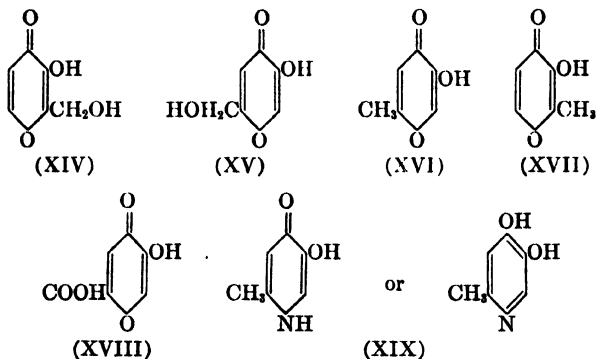
## VII. Kojic Acid

Saké is an alcoholic drink manufactured in Japan by the fermentation of steamed rice by a mixture of organisms of which strains of the *flavus oryzae* group of the aspergilli are perhaps the most important. The name koji is given to the mycelial mass thereby produced. Saito (124) found that this mold in pure culture produced in the rice an ether-soluble acidic substance of formula  $C_6H_6O_4$ . Yabuta (165) studied the substance and named it kojic acid. Kojic acid forms a diacetyl derivative and a dimethyl ether which, on hydrolysis with baryta, gives the methyl ether of acetol,  $CH_3COCH_2OCH_3$ , methoxyacetic acid, and formic acid. These reactions and the deep red color given with ferric chloride suggest the presence of a substituted  $\gamma$ -pyrone ring in kojic acid and would be explicable on either of formulas (XIV) or (XV). Allomaltol (XVI), a reduction product of kojic acid, is not identical with maltol, (XVII), a substance obtained from malt, pine needles, larch bark, and by hydrolysis of streptomycin (127). This excludes formula (XIV) for kojic acid.

Benzenediazonium salts and also nitrous acid (amyl nitrite and hydrochloric acid) react with kojic acid giving benzeneazo and isonitroso derivatives, respectively, indicating the presence of the group  $-C(OH)=CH \rightleftharpoons -COCH_2$ , which can only be reconciled with formula (XV). This conclusion is confirmed by the conversion of both kojic and comenic acids (XVIII) into the same methyldihydroxypyridine (XIX).

Challenger, Klein, and Walker (37) showed that a particular strain of the *A. flavus oryzae* group forms kojic acid not only from glucose but also from arabinose and xylose. Birkinshaw, Charles, Lilly, and Raistrick (18) observed the formation of kojic acid from pentoses several years earlier, but publication was delayed.

Tamiya (139) made an exhaustive study of the capacity of various organic compounds to yield kojic acid under the influence of *A. oryzae*. He determined the respiration figures and growth characteristics for these substances. From certain defined constants it was found possible to forecast the production or nonproduction of kojic acid, with very few exceptions.

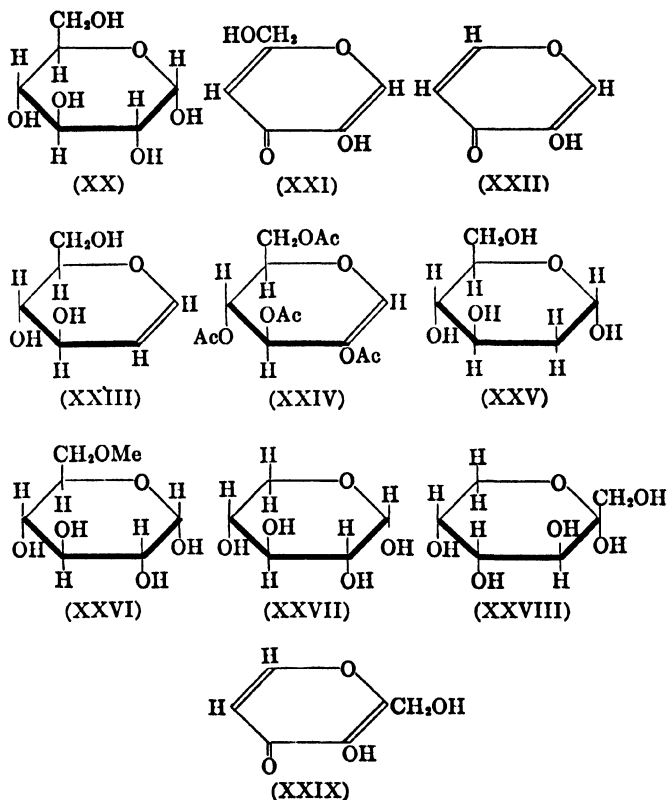


The similarity in structure between glucose (XX) and kojic acid (XXI) suggests, at first sight, that the conversion of (XX) to (XXI) involves simple loss of two molecules of water and oxidation of  $-\text{CH}(\text{OH})-$  to  $-\text{CO}-$ ; this so-called "carving-out" or non-fission process would be effected on the intact hexose molecule. According to this view, however, the pentoses arabinose and xylose (XXVII) should yield pyromeconic acid (XXII). Since kojic acid is actually produced, ring fission must first occur yielding probably a triose and a diose.

Challenger, Klein, and Walker (37) showed that dihydroxyacetone gives a 30% yield of kojic acid in cultures of *A. oryzae* and suggested that this might arise directly from two molecules of dihydroxyacetone by oxidation and loss of water without preliminary condensation to a hexose. They stated "the conversion of glucose to kojic acid may also be preceded by ring-fission and the hydroxy- $\gamma$ -pyrone nucleus may not be directly carved out of the pyranose ring." Corbellini and Gregorini (47) reached a similar conclusion following their observation that kojic acid is obtained by the action of the mold on fructose (XXVIII).

Fission of a pentose into a three-carbon compound and a two-

carbon compound has been observed recently by Kaushal and Walker (73), who detected glycolaldehyde during the formation of cellulose by *Acetobacter acetigenum* growing on media containing xylose or arabinose as sole source of carbon.



Barnard and Challenger (4) studied the behavior of several derivatives of carbohydrates in cultures of *A. oryzae* in the hope that evidence for or against the occurrence of the nonfission process might be obtained by the isolation of derivatives of  $\gamma$ -pyrone other than kojic acid. Pentaacetylglucose and tetraacetyl hydroxyglucal (XXIV), where fission was not to be expected, were completely metabolized, but no derivative of  $\gamma$ -pyrone could be isolated. The

mold did not grow on glucal (XXIII) nor on 6-methylglucose (XXVI). A 20% yield of kojic acid was obtained from 2-des-oxyglucose (XXV); here it is to be noted that fission could occur giving glyceraldehyde and  $\beta$ -hydroxypropionaldehyde. These results clearly afford no evidence in favor of a nonfission process for kojic acid formation.

Barnard and Challenger (3) have shown that when an inorganic nutrient medium containing 1.3 to 2.1% of ethyl alcohol as sole carbon source is inoculated with spores or placed under well-washed mycelia of *A. oryzae*, kojic acid is produced in yields of 12–17%. The mycelia produced no kojic acid on the alcohol-free medium. Acetaldehyde was detected in all cultures of the mold on ethyl alcohol (1 to 4%) whether or not kojic acid was produced.

As stated in a previous section, Gould (63) supplied evidence that the processes which give rise to kojic acid in a sugar medium do not involve the phosphorylating mechanisms of the Meyerhof scheme of alcoholic fermentation and the same author also showed that the reserve carbohydrates of the mycelium are not the source of the acid.

Apart from members of the *A. flavus oryzae* group and closely related aspergilli the only other mold found to yield kojic acid is *Penicillium daleae* Zal. (18). Claims have been made by Japanese workers (33,138) to have obtained kojic acid by the action of certain species of *Acetobacter* on fructose and on mannitol, but their cultures were not lodged in any available collection and the work is unconfirmed.

Kojic acid has been synthesized by Maurer and Petsch and a valuable discussion of the synthesis is given by Stacey and Turton (133).

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# PRINCIPLES OF ENZYMIC HISTO- AND CYTOCHEMISTRY

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## I. Introduction

Histo- and cytochemistry deal with the chemical constitution of cells and their components.

This new field extends beyond the old limitations of biochemistry—investigations of the organ, blood, and excretory products; and it offers to draw morphology out of the stage of mere architectural delineation by endowing the extra- and intracellular forms with the significance of their chemical nature and function. The actual course of progress in any such field is conditioned primarily by the development of instrumentation and methods. In the present case two main lines of endeavor stand out. The morphological techniques have led to the use of staining reactions which, when they can be relied upon, enable rather fine enzymic localizations of a qualitative nature. On the other hand, the quantitative techniques are not usually capable

of the finer localizations *in situ*. Obviously, it would be preferable to establish both the quantitation and the topographical disposition of these biologically significant components. While this goal cannot often be attained today, and can be approached in a few instances only by a combination of techniques, the continuing efforts toward this end hold much promise.

It is not the purpose of the reviewer to discuss the many and diverse special applications of enzymic histochemical methods, an undertaking manifestly impossible in the space allowed; neither will details of methodology be presented, since these have recently been given elsewhere (55), but the principles of the methods and their scope will be discussed.

## II. Microscopic Techniques

### A. USE OF STAINING METHODS

Staining methods for the identification of chemical substances, groups, or activities in tissue and cellular structures have been developed primarily by the morphologists, who adapted certain chemical identifying reactions to their standard histological techniques. Unfortunately, the great majority of the staining methods described are of little value due either to their lack of chemical specificity or their failure to effect true localizations. The problem is indeed a difficult one, as is apparent when one considers the requirements to be met. These have been listed by the writer (55) as:

(1) Preparation of microtome sections in which there has been no significant alteration in the position of the constituent being investigated. (2) A reagent which is specific for this tissue constituent. (3) A reaction between the reagent and constituent which is of such a nature, and rapid enough, to obviate diffusion of the constituent or of the reaction product. (4) A reaction product, thus trapped *in situ*, which is capable of being visualized.

For the demonstration of the disposition of enzymes, the following requirements must also be added:

(5) A reagent which when added to the buffered substrate will react with one of the enzymic products but not with the substrate or buffer. (6) A reagent which will also have no untoward effect on the enzyme. (7) If the enzymic product which reacts with the reagent is a substance pre-existing in the tissue, either the sites of enzyme action must be different from those of the pre-existing sub-

stance, or the increase in the amount of the visualized compound resulting from the enzyme activity must be demonstrable, or, better yet, the substance must be removed in advance by a method which will not take out the enzyme. (8) A control experiment in which either the substrate is omitted, or a highly effective soluble enzyme inhibitor, such as fluoride, is added (the inhibitor must not react with substrate, buffer, reagent, or products). The advantage of the inhibitor is that, in some cases, naturally occurring substrate may be present with the enzyme and thus give a false aspect to the nonenzyme control. No such control experiment is required if all substances pre-existing in the tissue and capable of giving a positive reaction can be removed without seriously reducing the enzyme activity. This has been accomplished in certain tissues for the phosphatase test.

Since very few of the methods employed meet all these requirements much of the published work is not only of little value but it is often actually misleading. The greatest difficulty arises in attempts to visualize constituents *in situ* which are highly diffusible, *e.g.*, inorganic ions. Fortunately, enzyme diffusion is considerably less, and hence greater reliability in cellular localizations may be obtained, although very real dangers from adventitious adsorptions and translocations still exist.

Fixation, when such treatment does not entirely destroy the enzyme activity, aids in maintaining its localization. The more preferable procedure, however, is the freezing-drying method previously discussed (55), which enables one to minimize diffusion by holding the fresh tissue in a frozen state until exhaustively dehydrated, and which avoids the use of any liquid other than the molten paraffin used in the embedding process. However, once the paraffin sections are so obtained, they must be deparaffinized and treated with various solutions to bring about the reactions necessary for visualization. Thus the danger of diffusion is still not eliminated, but it is reduced considerably, often to a negligible factor, and the freezing-drying procedure is certainly the method of choice. If one could avoid the use of solutions entirely by employing gaseous reagents, the dislocations resulting from liquid treatments could theoretically be obviated. However, to the writer's knowledge, this has not yet been attempted.

The fallacy of employing the intensity of an enzyme stain as a

quantitative estimate of the activity in the tissue should be obvious, particularly since the activity remaining is usually a small part of the original as a result of the insults of the staining technique. Nevertheless, there are those who try to make interpretations on the basis of the intensity of the color developed. This is a doubtful practice even when a particular substance, rather than an activity, in the tissue is being visualized, since uncontrolled variables usually operate in even the simplest histological staining routine.

Staining methods have been reported for oxidative enzymes. The methods depend on the development of colored compounds by the products of the enzyme actions. However, the colored compounds are usually soluble in the medium in which they are formed, and hence their diffusibility seriously limits the proper localization of these enzymes.

**Peroxidase.** The peroxidase methods of Armitage (8) and McJunkin (124) depend on the formation of an initial blue color which turns to brown when the enzyme acts upon a solution of benzidine in 30–40% methyl alcohol containing hydrogen peroxide.

**Dopa Oxidase.** The procedure of Bloch for dopa oxidase has been adapted by Laidlaw and co-workers (92,93) for histochemical use. Dopa solution buffered to pH 7.4 by phosphate is employed as the substrate and the enzyme action is made apparent by a blackening due to the formation of melanin. Characteristics of the reaction were discussed by Sharlit *et al.* (142).

**Amine Oxidase.** Amine oxidase has been demonstrated in tissue sections by Oster and Schlossman (132), who visualized the aldehyde formed by the enzymic oxidation of tyramine. The fuchsin-sulfurous acid Feulgen reagent was used to develop a blue color with the aldehyde, and preformed tissue aldehydes and "plasmal" were prevented from interfering by binding them with bisulfite before applying the substrate solution.

**Cytochrome Oxidase.** Histochemical methods for the enzymic oxidation of a mixture of *p*-aminodimethylaniline and  $\alpha$ -naphthol to indophenol, or of *p*-phenylenediamine to the diimine, in the presence of cytochrome *c*, may be found in the works of Lison (119) and Glick (55). The blue or blue-violet color obtained is used as an indication of the presence of the enzyme.

**Succinic Oxidase.** Semenoff (140) claimed that succinic oxidase could be demonstrated in tissue sections by the reduction of methyl-

ene blue in a mixture of the dye and succinate solution. The fading of the dye in regions of enzyme activity was taken to indicate the presence of the latter.

Methods have also been elaborated for urease, phosphatases, zymoexase, lipase, and cholinesterase. With the exception of the urease technique, these methods have met with some success and therefore will be discussed in more detail.

**Urease.** A urease method described by Sen (141) and used by him for a study on the jack bean depends on the detection of the carbon dioxide formed by the enzymic decomposition of urea. The carbon dioxide is converted to a precipitate of either calcium or cobalt carbonate at the site of its liberation by the presence of calcium or cobalt ions in the substrate medium. The calcium salt is converted to silver carbonate and then to black metallic silver; or if the cobalt salt is used it is converted to black cobalt sulfide for easy visualization. Both Gomori (59) and Schwartz and Glick (139) have found the Sen method unsatisfactory for mammalian tissues and have attempted to improve it without sufficient success.

**Alkaline Phosphatase.** The general principle of the Sen method was used successfully by Gomori (60) and Takamatsu (150) for the localization of alkaline phosphatase in tissue. The phosphoric acid liberated from glycerophosphate by the enzyme is trapped *in situ* by calcium ions present in the solution, and the calcium phosphate is then converted either to silver or cobalt phosphate and finally to the black metallic silver or cobalt sulfide, respectively. The cobalt, rather than silver salts, are now more commonly used.

The decalcification processes usually employed for hard tissues destroy the phosphatase so that the staining of these tissues poses a special problem. Kabat and Furth (88) found that 10% diammonium citrate would effect certain decalcifications without destroying phosphatase. Since cobalt sulfide is insoluble in trichloroacetic acid, Bourne (24) proposed that small pieces of bony tissue be treated in the usual manner for the demonstration of alkaline phosphatase and, after the deposit of cobalt sulfide has been formed, decalcification by trichloroacetic acid be performed as a final step. Bourne (24) also employed sodium alizarin sulfonate in Gomori's substrate medium to visualize the calcium phosphate produced by automatically staining it red as it is formed.



Activation of the phosphatase by magnesium ions was employed in the procedure of Kabat and Furth (88) and subsequently in a revised method by Gomori (64). Activation of the phosphatases in bacteria by magnesium-alanine and manganese-alanine mixtures was used in a study by Bayliss, Glick, and Siem (13).

The detection of the organic, rather than the phosphate moiety of the substrate was the basis of another method presented by Menten, Junge, and Green (127). Calcium  $\beta$ -naphthol phosphate was employed as the substrate and the  $\beta$ -naphthol liberated by the enzymic scission was made to react immediately with diazotized  $\alpha$ -naphthylamine, which was present in the substrate solution, to precipitate a reddish-purple dye. The advantage of this method is that the presence of phosphate in the tissue will not give a false positive reaction, but the disadvantage of its more involved procedure is in itself sufficient to make it a less desirable method since the problem of the phosphate in tissue can be circumvented in most cases by prior removal and suitable control experiments (55).

The specificity of the stain has been demonstrated by Gomori (60,62) and Kabat and Furth (88). After a critical study of each step in the Gomori method, Danielli (39) concluded that the localizations obtained are reliable; this investigator also examined the method of Menten *et al.* and found the technique not as satisfactory for low enzyme activities as that of Gomori. The work of Emmel (51) also lends weight to the reliability of the Gomori method. A study of the loss of activity attending fixation and paraffin embedding was carried out by Stafford and Atkinson (146), who reported that 20–30% of the original activity remained in the embedded tissue and that alcohol fixation resulted in less destruction of the enzyme than acetone fixation. Danielli (39) had previously found that, whereas alcohol and acetone were "fair" fixatives, 4% formaldehyde in either 1% acetic acid or 70% pyridine, or 20% pyridine in 70% alcohol were "very good."

**Acid Phosphatase.** The alkaline phosphatase method of Gomori cannot be directly adapted to acid phosphatase because of the solubility of calcium phosphate at the lower pH values required. However, by employing lead instead of calcium ions at pH 4.7, and by subsequent conversion of the lead phosphate to lead sulfide, Gomori (61) was able to visualize the acid phosphatase as a brown or a black deposit. Improvements were introduced by Wolf, Kabat,

and Newman (157); and Moog (129,130) showed that manganese ions or ascorbic acid in the substrate medium would activate the enzyme. After considerable experience with the method Gomori (64) revised his original procedure.

The level of success with which the alkaline phosphatase method has been applied has not been attained by this acid phosphatase method. Gomori (64) stated: "For some unknown reason, the staining for acid phosphatase sometimes turns out patchy, occasionally even negative, when it should be positive. This seems to happen especially in cases when the pieces have been exposed to the temperature of the paraffin oven for more than an hour, or when the temperature of the oven is over 56°C." Both Lassek (95) and Bartelmez and Bensley (10) observed that the staining of nervous tissue would occur in spite of drastic chemical and heat treatments, which undoubtedly destroyed all enzyme activities, and it was concluded by both groups that the method lacked the specificity required to make it worth while as a stain for acid phosphatase in this type of tissue.

With the staining reactions for both forms of phosphatase, false positive staining will occur not only in the presence of preformed phosphate but also in the presence of anything else which can be precipitated by the calcium or lead ions under the conditions employed. Neither Lassek nor Bartelmez and Bensley treated the sections to remove interfering substances prior to application of the substrate medium. For certain tissues (55) it has been found that all such pre-existing substances capable of giving a false positive stain can be removed by 0.1 *M* citrate buffer, pH 4.5 to 5.0 (64). However, even if this treatment were applied, false positive staining for the enzyme might still occur since Bartelmez and Bensley (10) showed that the staining reaction can be elicited even in boiled sections at sites of injury to the tissue. Factors which can cause a liberation of phosphate from any of its many organically bound forms in the tissues would also result in staining, quite apart from the specific effect of the phosphatase on the substrate solution. In fact, this would also be true of the liberation of any other substance, *e.g.*, long chain fatty acids, carbonates, sulfates, etc., capable of precipitation with the calcium or lead ions under the experimental conditions used for the alkaline and acid phosphatases, respectively. It is essential, therefore, that any treatment employed for the de-

struction of the enzyme for purposes of experimental control be such as to prevent the liberation of these interfering substances. Another method which might circumvent some of the difficulties would be application of a stain for the organic rather than the phosphate product of the enzymic scission. This principle was followed by Yin (158) for work on plant tissues; he used the method of Menten *et al.* (127).

The literature is cluttered with publications in which the results of enzyme stain work have been naively presented as unequivocally reliable, without thought or comment concerning the attendant factors serving to distort the true picture.

**Other Phosphatases.** By substitution of other substrates in the media employed for the alkaline and acid phosphatases, other phosphatase enzymes have been studied by the staining technique. Thus Wolf, Kabat, and Newman (157) used both ribonucleic acid and glucose-1-phosphate in their work on nervous tissue. For studies on duodenal tissue, Dempsey and Deane (45) and, on thyroid tissue, Dempsey and Singer (46) employed adenylic acid, ribonucleic acid, glucose-1-phosphate, fructose diphosphate, and lecithin as substrates. Glick and Fischer (57,58) used adenosine triphosphate, thiamine pyrophosphate, and glucose-1-phosphate as substrates in an investigation on wheat, and Krugelis (91) used adenylic, guanylic, cytidylic, ribonucleic, and desoxyribonucleic acids in a study of the salivary glands of *Drosophila* larvae.

Difficulties arise in certain instances when more than one enzyme can act on the same substrate. Thus alkaline phosphatase and adenylypyrophosphatase (adenosine triphosphatase) both can act on adenosine triphosphate, and both phosphatase and phosphorylase can liberate phosphate from glucose-1-phosphate. In order to distinguish between the enzymes in such cases it is necessary that their cellular localizations be different, that differences in properties such as pH optima be exploited, or that the organic moiety be visualized. In the case of phosphorylase, a stain for the polysaccharide was employed by Yin and Sun (159).

**Zymohexase.** The enzyme system which converts hexose diphosphate to dihydroxyacetone phosphate and phosphoglyceraldehyde has been subjected to the general technique for the localization of phosphatases by Allen and Bourne (2). Iodoacetate was added to

the substrate medium to prevent enzymic breakdown of the triose phosphates which spontaneously liberate inorganic phosphate at room temperature in alkaline solution. Phosphate thus set free was visualized by the Gomori method. In the muscle tissue studied, Allen and Bourne claimed that a distinct difference in localization of the zymohexase and alkaline phosphatase obviated mutual interference, and they also reported that fluoride could be used selectively to block the phosphatase activity. Paraffin embedding destroyed the activity, so that frozen sections were used.

**Lipase.** The principle of the phosphatase method was also used by Gomori (63,65) for the localization of lipase. Commercial water-soluble esters of long chain fatty acids, Product 81 (Onyx Oil & Chemical Co., Jersey City, N. J.) or Tween 40 or Tween 60 (Atlas Powder Co., Wilmington, Del.), were employed as substrates. The fatty acid liberated by the enzyme was precipitated by calcium ions in the substrate solution and the eventual conversion of the calcium salt to lead sulfide was carried out as in the alkaline phosphatase procedure.

**Cholinesterase.** Gomori (66) has also employed choline esters of high molecular weight fatty acids as substrates for the visualization of cholinesterase. The method is a variation of that for lipase.

## B. USE OF ENZYMES AS REAGENTS

Attempts have been made to localize certain intra- and inter-cellular constituents capable of selective enzymic decomposition by comparing sections stained for the constituents before and after the enzyme treatment. In this connection work on the action of trypsin and ribo- and desoxyribonucleases on chromosomes has been reviewed recently by Catchside and Holmes (27), and the use of enzymes for the localization of nucleic acids in the cell, malignant tissue, and embryo was summarized by Davidson (42,43), Stowell (149), and Brachet (25), respectively. The use of amylase to remove glycogen is one of the older methods (16,99). Several attempts are being made currently in different laboratories to employ hyaluronidase preparations for the removal of hyaluronic acid from tissue sections.

Criticism of the use of enzymes for histochemical localizations has

been advanced by Danielli (41), who pointed out that this method presupposes that the enzymes used will act only on the particular substance being investigated, and that the enzyme will be accessible to the substrate in the tissue. Attempts to deal with the first requirement have led to the use of highly purified or crystalline enzymes but, as Danielli points out, there is no way known of proving that an enzyme preparation will act only on a given substrate in the tissue. Accessibility of the enzyme to the substrate constitutes another problem. Danielli presented the interesting situation: "A monolayer of protein over nucleic acid would probably suffice to prevent access of enzymes to the nucleic acid. The paradoxical situation therefore arises that, if pure ribonuclease were available, it could destroy a cell organ consisting mainly of protein held together by an almost negligible ribonucleic acid matrix, whereas an organ composed mainly of nucleic acid, but held together by a matrix of protein, could be left intact. It is at this point that one begins to wonder whether the 'successful' use of enzymes in cytology has not depended on the impurity as much as on the purity of the enzymes." The conclusion of Danielli was: "Consequently, promising as this method is in theory, its hazards are so great that I have not thought it profitable to use."

The present writer is of the opinion that, while the difficulties inherent in this technique are indeed very serious, they should not be allowed to paralyze efforts toward their mastery. But the interpretation of the findings obtained through the technique should include a critical awareness of its limitations. As long as a method, no matter how imperfect, can offer some information not attainable otherwise, it should be seriously pursued, and applied with discretion.

### III. Quantitative Techniques

#### A. OBTAINING THE ANALYTICAL SAMPLE AND ITS QUANTITATION

As stated in the introduction, the quantitative techniques usually do not enable the finer localizations of cellular constituents to be made. The limitation operative, as a rule, is the degree to which the anatomical entities can be isolated for their separate analyses. Commonly used procedures for this purpose are microtome sectioning of fresh frozen tissue, isolation of cells or cellular particu-

lates by centrifugation, or microdissection. It is necessary, of course, to know the morphological composition of the sample employed so that a correlation between structure and chemistry can be made.

### 1. Microtome Sectioning

In rather rare instances a given cell layer can be separated cleanly from adjacent tissue without the use of special apparatus. Thus in studies on carcinogenesis in mouse epidermis, Cowdry's group at Washington University has developed a simple technique for the separation of the epidermis from dermis by blunt dissection after warming (12). More often, however, the cells are not so conveniently separated and sectioning with a microtome is necessary.

In the microtome work, a section adjacent to the one used for analysis can be employed for histological identification by the usual staining procedures. In most tissues the cellular composition of adjacent sections will not be greatly different. However, in tissues in which this is not so, as in the case of the retina, which has histological changes every 40 to 50  $\mu$ , it is necessary to carry out the analysis and the microscopy on the same section. A method for accomplishing this was given by Anfinsen *et al.* (7) who first stained the section with a nonaqueous solution of methyl violet, examined it and then used the section for analysis. Anfinsen and co-workers reported that the staining treatment had no significant effect on the peptidase or diphosphopyridine nucleotide of rat liver, or on the cholinesterase of rat brain. In some cases it is possible to identify the section histologically by inspection without staining. This was the procedure used by Linderstrøm-Lang and Engel (107), who employed photomicrography to record the histological pattern of fresh barley sections prior to their use for amylase determinations.

### 2. Centrifugal Methods

The use of centrifugation techniques for the separation of cellular components has been acquiring an increasing popularity. This usage can be divided into separations in single cell organisms, *e.g.*, the sea urchin egg, and in tissue homogenates. While it is necessary to employ microchemical methods for enzyme studies in the former instance, it is usually possible to apply macromethods in the latter, since relatively large quantities of homogenate can be handled.

As Danielli (40) has properly cautioned, the centrifugal segregation of cellular particulates could possibly affect the enzyme activities associated with the particulates *in situ*. Naturally occurring enzyme inhibitors or activators might be displaced by the disturbance of the original organization of the cell. Hence it would be well to employ control experiments designed to test for effects of this nature by judicious recombinations of separated fractions.

**Separations in Single Cell Organisms.** The centrifuge microscope of E. N. Harvey and A. L. Loomis has assisted in the development of the separations by enabling direct observation of the cells at high magnification while they are being subjected to the centrifugal force (72,73). A technique was developed by Harvey (71,72) for the centrifugation of cells in an isosmotic medium having a density gradient. For studies on *Arbacia punctulata* ova, sea water was layered over 0.95 molal sucrose solution, and at the interface the liquids were mixed to form a more gradual gradient. When sufficient centrifugal force (10,000 times gravity) was applied, internal stratification, elongation, and eventual scission of the eggs in this medium occurred (68,69). The composition of the solutions making up the density gradient has been varied to meet particular requirements. Thus for the amoeba, Holter and Doyle (81) employed a 20% solution of gum arabic underlying the culture medium. Staining with vital dyes is employed at times to visualize certain of the formed bodies (3-5,70).

Micrurgical techniques may be used to obtain single cells, and in some instances to dissect them or remove their formed bodies. The micromanipulator and its use for this purpose have been described in reviews by Chambers and Kopac (28), and by Kopac and others in the volume edited by Reyniers (134). It is often of value to combine the centrifugal technique with microdissection to obtain material for enzyme investigations, *e.g.*, the studies of peptidase in parts of the amoeba (83).

**Separations in Tissue Homogenates.** Methods of dealing with tissues usually involve a different treatment from that used for single cell work. When a tissue consists primarily of one type of cell, it is possible in certain cases to disrupt the cells by homogenization, and to apply differential centrifugation for the separation, first, of nuclei and cytoplasm, and then of the nuclear and cytoplasmic particulates.

**Nuclei Separations.** Ever since Miescher (128) digested leucocytes with pepsin to remove the cytoplasm, attempts at the isolation of nuclei have followed diverse lines. Disruption of the avian erythrocyte by laking in distilled water (1), treatment with lysolecithin (94), with saponin (50), and freezing and thawing (152) have been used prior to centrifugal isolation of the nuclei. The freezing-thawing method often results in partial agglutination and damage to the nuclei.

Mechanical disruption of tissue followed by treatment with citric acid, before the centrifugations, was employed by Crossmon (37) for the separation of muscle cell nuclei; and Stoneburg (148) added pepsin in a modification which he applied to leucocytes, and muscle and tumor cells. The citric acid method was altered by Dounce for use on liver (47,48) and tumor (49) cells. A method without citric acid, and less drastic in other respects as well, was employed by Lazarow (97,98) and Hoerr (77) in their work on liver cells.

Behrens (14) introduced the principle of nuclei separation by centrifugation in nonaqueous media (benzene-carbon tetrachloride mixtures) of controlled specific gravity, which he applied to heart muscle, and later to thymus. The method was also used for rye germ by Feulgen, Behrens, and Mahdihassan (52) and for liver by Behrens (15). A modified Behrens procedure was employed by Mayer and Gulick (126) to obtain nuclei from thymus cells, and by Williamson and Gulick (156) for cells from tonsils and lymph glands. In general, the use of nonaqueous media is drastic for many purposes, and the procedure is rather laborious.

By taking pains to maintain low temperature during all operations, Claude and Potter (36) succeeded in isolating chromatin threads from the nuclei of spleen and liver cells. The treatment was made as gentle as possible and involved only grinding the tissue and differential centrifugations in physiological saline solution.

**Cytoplasmic Separations.** Isolation of mitochondria has followed the procedure developed by Bensley and co-workers (17,18, 77) in their work on the liver cell. The entire procedure is performed at 0°, and it involves gentle grinding, kneading through bolting silk, suspension in physiological saline solution, and differential centrifugation. A modification was employed by Claude (33)



and Claude and Fullam (35) for certain neoplastic cells, and by Claude (34) and Hogeboom *et al.* (78) for liver cells.

Claude (30) discovered and separated submicroscopic particulates, which he later termed "microsomes," in saline extracts of embryonic chick tissues. Subsequently microsomes were separated from a large variety of tissues by Claude (32). These particulates were obtained as a jellylike pellet by subjecting the supernatant fluid, remaining after sedimentation of mitochondria at 2400, to 18,000 times gravity. By applying a centrifugal force of 95,000 times gravity to the supernatant from the microsome separation, Barnum and Huseby (9) obtained the sedimentation of another fraction as a transparent red pellet. In an as yet unpublished study, these investigators separated the microsome fraction into "lipoprotein" and "nucleoprotein" portions.

A submicroscopic particulate of glycogen was isolated from guinea pig liver by Lazarow (96,97) by subjecting the supernatant material from the mitochondria separation to preliminary clarifications at 6000 r.p.m. in an 8 inch angle centrifuge, followed by sedimentation at 12,000 r.p.m. The packed glycogen at the bottom of the tube was covered with a red layer of mixed glycogen and lipoprotein particles which could be washed off. If the glycogen were removed from the red material by digestion with diastase, the lipoprotein could be sedimented as a transparent gelatinous pellet.

Differential centrifugation has also been employed to separate other particulates such as melanin (31), and ferritin, conjugated proteins, bacteriophage, and fowl tumor virus (147). Chloroplasts have been isolated from leaves by the centrifugation technique (67,131). It was also employed by Henle *et al.* (76) and Zittle and O'Dell (163) for the separation of the heads, midpieces, and tails of spermatozoa disrupted by sonic vibrations.

### 3. Quantitation of Sample

The definition of the quantity of the separated sample often poses a difficult problem. Weight, volume, numbers of cells, and nitrogen or nucleic acid content have been variously employed for this purpose.

**Weight.** Commercial microbalances can be used for the measurement of the weights in some instances, but the lower range of the

quartz fiber balance developed by Lowry (120,121) and later modified by Kirk *et al.* (89) is considerably more sensitive and better adapted for the direct weighing of the very small masses to be handled. The ingenious use of the Cartesian diver as a balance has been described by Zeuthen (162), and Levi and Zeuthen (100) also employed the gradient tube for microweighings. Linderstrøm-Lang and Holter (111) employed the "reduced weight," a quantity having the advantage of affording a measure of the amount of a tissue sample independent of its water content. The "reduced weight" is defined as the weight of the sample minus the weight of an equal volume of water, and it can be measured in the gradient tube to be described later under dilatometry. Levi and Zeuthen (100) subsequently made a thorough study of this measurement.

**Volume.** The volume of microtome sections cut from a circular block of tissue can be calculated easily from their thickness and diameter. More accurate sectioning of given thickness can be accomplished by placing the entire microtome in a cryostat as described by Linderstrøm-Lang and Mogensen (116), thus avoiding the variations occasioned by the intermittent cooling obtained when the ordinary freezing head is employed. The volume of irregularly shaped samples can be determined by a colorimetric dye method developed by Holter (80), and in some cases, *e.g.*, *Amoeba proteus*, it is possible to draw the object into a capillary tube of known diameter, measure its length, and compute its volume (125). If the body is spherical, the volume is usually calculated from the diameter, which can be measured easily under the microscope with a micrometer attachment.

**Numbers of Cells.** A technique for counting each type of cell in histological preparations was described by Linderstrøm-Lang, Holter, and Sjøeborg Ohlsen (113), and it was used by them in ascertaining enzyme activities per cell in the gastric mucosa. Later Rask-Nielsen (133) extended some of these studies.

**Nitrogen and Nucleic Acids.** Many methods for the measurement of nitrogen in microtome sections or other histochemical samples have appeared. These have been recently reviewed (55) and the most reliable of them is that of Brüel, Holter, Linderstrøm-Lang, and Rozits (26), which includes improvements developed over a long period of critical trial. Berenblum, Chain, and Heatley (19) used the phosphorus content of tissue as a measure of the

nucleic acid after the acid-soluble and lipide phosphorus were removed.

#### B. USE OF COLORIMETRIC AND TITRIMETRIC METHODS

**Colorimetric Methods.** Once the sample is obtained, the analytical treatment that is applied for enzyme measurement differs from its macro counterpart chiefly with respect to the volumes employed and the instrumentation required. Usually the same reactions and concentrations of reagents are used in both the micro- and macro-methods, and the reliability and accuracy of the former are of the same order as those of the latter, from which they are adapted.

Colorimetric methods adjusted to the scale required for most quantitative histochemical work have been those concerned with the analysis of inorganic and organic substances but not specifically with enzyme measurements, except in the case of phosphatase. A method of measurement of this enzyme by visual colorimetry in capillary glass tubes was described by Weil and Russell (154). Their measurements were confined to plasma, of which less than 1  $\mu$ l. was required for determinations giving an average deviation of  $\pm 3.0\%$ . But the use of microcuvettes in standard photometers enabled Siwe (143), Lundsteen and Vermehren (123), and Bessey, Lowry, and Brock (20) to employ a more objective estimation of the intensity of the color developed in phosphatase procedures using small volumes. With standard phosphomolybdate methods, Siwe, and Lundsteen and Vermehren used cuvettes of 0.2 ml. capacity with the Pulfrich step photometer (Zeiss). With chromogenic substrates, Bessey *et al.* employed the cuvette of Lowry and Bessey (122) in the Beckman quartz spectrophotometer. The cuvette can be used for volumes of 0.05 ml. or less, and in their work on serum only 5  $\mu$ l. was required for colorimetry on 0.5 ml. of final solution. While all of these investigators were concerned with blood analysis, adaptation of the method to measurements of cellular phosphatase should offer no great problem with any of the reliable methods. Kirk *et al.* (89) described a plastic body capillary absorption cell which can also be used with the Beckman instrument. With an internal diameter of 2 mm. and a 5 cm. light path the capacity is about 160  $\mu$ l.

Undoubtedly, the advent of microcuvettes will stimulate the use of other colorimetric methods for histo- and cytochemical investi-

gations on enzymes. The cuvette of Lowry and Bessey is made with quartz windows so that absorption in the ultraviolet may also be recorded.

**Titrimetric Methods.** The development of the titrimetric methods was primarily initiated by Linderstrøm-Lang, Holter, and co-workers at the Carlsberg Laboratory in Copenhagen. The enzymes for which methods have been described are carbohydrases (81,107,110), peptidases and proteases (85,106,109,144,153), simple esterases and lipases (53,56), cholinesterase (54), arginase (118), urease (111,117), and catalase (82,86). The apparatus manipulation, and detailed procedures, which have been described elsewhere (55), involve no essentially new principles beyond the use of apparatus required to deal with the small volumes (usually about 1000 times less than those in macromethods), such as microburettes, micropipettes, etc.

### C. USE OF GASOMETRIC METHODS

The ordinary Warburg or Barcroft apparatus has been used for histo- and cytochemical investigations in some cases. Thus, when a tissue is composed primarily of one type of cell the more macro devices may serve very well, *e.g.*, adenylypyrophosphatase measurements on mouse epidermis (135), or when a number of cells of the same kind can be pooled, *e.g.*, tyrosinase studies on grasshopper eggs (21). Of course, studies have also been made on tissues composed of several types of cell when the material can be separated in a clearly defined histological form.

The advantages of dealing with the single cell and its anatomical components for cytological studies are obvious. The more practical gasometric techniques developed to meet this need have fallen into three main categories: volumetric capillary respirometry, Cartesian diver manometry, and polarography. It should be mentioned in passing that Heatley and co-workers (74,75) developed an optical lever respirometer in which the respiration chamber is covered with a mica membrane to which mirrors are attached. A change in gas pressure within the chamber causes the mica to bulge and a compensating external pressure can be applied to restore the membrane to its original position as indicated by an optical lever system. From the volume of gas space and the pressure change required to keep it constant, the change in gas volume can be calculated. The

apparatus is used for changes in gas volume of about 1  $\mu$ l. per hour. The instrument is much more complicated, mechanically, than the other ones mentioned, and it is less sensitive.

### *1. Volumetric Capillary Respirometry*

As in other volumetric apparatus for gasometric measurements, the microinstrumentation is designed to enable direct measurement of gas volume at constant pressure. The volume of the apparatus need not be determined, as it must for manometric apparatus. In principle, changes in the gas volume in a capillary tube connected to a respiration chamber are followed by the position of an index droplet placed across the lumen of the capillary. One end of the capillary may be open to the air, in which case corrections for barometric changes would have to be made, or a differential instrument may be used in which both ends of the capillary are connected to chambers in a closed system. If both chambers are made in a block of heat-conducting material, and if they are of about the same size, temperature fluctuations will have no significant effect beyond that on the reaction rate itself. The actual displacement of the index droplet would be only about half that in the open capillary instrument. When the compensating chamber is made very large with respect to the reaction chamber, the displacement of the index droplet in the differential instrument can be made to approximate that in the open type. Temperature variations would more gravely affect the instrument with a large compensation chamber and hence would require careful control.

An open capillary respirometer was described by Cunningham and Kirk (38) which had a sensitivity of  $5 \times 10^{-5}$   $\mu$ l. Although its absolute accuracy is in doubt, relative accuracies were given as  $\pm 15\%$ . The technical difficulties in the use of this instrument will probably make it less desirable than one of the differential type. Of the latter, instruments have been constructed by Barth and Kirk (11), who employed chambers of approximately the same size (gas changes of the order of 0.1 to 10  $\mu$ l. per hour), Tobias and Gerard (151), who used a large compensation chamber (0.03 to 0.06  $\mu$ l. per hour), and Scholander (138), who employed his micrometer burette to measure the oxygen uptake by adding oxygen from the burette to maintain a constant gas volume in the presence of alkali used to

absorb  $\text{CO}_2$  evolved. One of the latter respirometers was sensitive to about 0.01 and another to about  $0.30 \mu\text{l.}$  per hour. The range of any of these can be varied somewhat by suitable alterations in the dimensions of the instruments. The respiration of single protozoa has been followed in capillary respirometers.

## 2. Cartesian Diver Manometry

The original application of the Cartesian diver to the measurement of volume changes in small volumes of gas was made by Linderstrøm-Lang (102). A short discussion of the technique appeared in a paper by Linderstrøm-Lang and Glick (108), and modifications were subsequently described by Boell *et al.* (22,23) and Rocher (136,137). The theoretical aspects have been thoroughly treated by Linderstrøm-Lang (103,104) and Linderstrøm-Lang and Holter (112); Holter (79) has presented a finely detailed description of the improved apparatus and technique incorporating the newer refinements developed at the Carlsberg Laboratory.

The principle of the instrument is based on the fact that any quantitative gas change occurring in the diver, which is used as the reaction vessel, requires a corresponding change in pressure applied to the surface of the liquid, in which the diver is submerged, to hold the gas volume in the diver constant so that it will remain submerged at a given level. Thus the changes in the external pressure applied become a measure of the changes within the diver.

The volume of the divers usually employed is  $5\text{--}10 \mu\text{l.}$ , but Zeuthen (160) has designed "capillary" divers having a gas volume of  $0.04$  to  $0.11 \mu\text{l.}$ , which he was able to use to follow respiration rates of  $2 \times 10^{-4}$  to  $2 \times 10^{-3} \mu\text{l.}$  oxygen per hour with an error of about  $2 \times 10^{-5} \mu\text{l.}$  per hour. Zeuthen suggested that for rates of less than  $10^{-2} \mu\text{l.}$  oxygen per hour the capillary diver be used, while for higher rates the larger divers be employed. For work on the respiration of frog eggs, a special diver was used by Zeuthen (161); a theoretical treatment of the diffusion problems posed by the measurement of periodic gas changes in this diver was presented by Linderstrøm-Lang (105). In addition to its use as a respirometer, the Cartesian diver has thus far been adapted to the gasometric measurement of cholinesterase (108), thiamine and cocarboxylase (155), and diphosphopyridine nucleotide (6).

### 3. Polarography

The application of polarographic technique to measurements of respiration on a histochemical level has been described by Davies and Brink (44). Two forms of stationary platinum microelectrodes have been developed which enable the determination of oxygen tension in animal tissues with a spatial resolution of 25  $\mu$ . The methods depend on the measurement of the current-potential relationships which accompany the reduction of dissolved oxygen at the platinum electrode. Under the conditions employed, it appears that no substances other than oxygen are electrolyzed. When a known potential is applied and the circuit is closed, the current falls as the oxygen concentration gradient spreads from the electrode wire into the surrounding solution. The current is recorded after a fixed number of seconds from the time the circuit is closed. Repetition of current measurements at various potentials are used to obtain data for the construction of a current-voltage curve from which the oxygen concentration can be determined in the manner customary in polarographic methods.

One type of electrode consists of the end of a platinum wire recessed in a cylindrical glass tip. Measurements of the oxygen tension cannot be made oftener than once every five minutes with this electrode because they depend on the attainment of a preliminary equilibrium between the oxygen inside and outside of the recess. The larger the recess, the longer the time required between readings. The recessed electrode has the advantages of freedom from convection effects outside the recess and independence of the oxygen diffusion coefficient in the medium beyond the orifice of the recess. Maintenance of a constant oxygen diffusion coefficient within larger recesses requires that the cavity be filled with salt-agar; experiments have also been conducted on electrodes whose orifices are covered with collodion films.

The other form of electrode is an open type in which a platinum wire, 25  $\mu$  in diameter, is fused into a glass tube so that one end of the wire is flush with the sealed end of the tube. The direct exposure of the wire to the oxygen-containing medium enables continuous recording of relative oxygen tensions. This electrode is rather unstable in performance, reproducible to only about 15% under favorable conditions, and it cannot be used for measurement of absolute oxygen tensions; but rapid changes in tension can be

followed. Thus, Davies and Brink recorded to about 0.1 second the sudden oxygen uptake accompanying the contraction of a muscle fiber.

Improvement in the apparatus and an extended applicability can be expected from the continuing studies in Bronk's laboratory. Among other studies, this group has been investigating the oxygen consumption of nerve cells *in situ*.

#### D. USE OF DILATOMETRIC METHODS

The principle of dilatometry is based on the phenomenon that chemical reactions in liquid systems are accompanied by isothermal changes in volume, which can be used in some cases as measures of the extent of the reactions. The utilization of this principle on the macro scale has been rather limited (145), and its adaptation to histo- and cytochemical problems by Linderstrøm-Lang (101) has been confined thus far to studies on peptidase (84,101,115), and ribonuclease (29). The macrodilatometric work on trypsin and chymotrypsin by Linderstrøm-Lang and Jacobsen (114) and Jacobsen (87) should be mentioned.

In the micromethod of Linderstrøm-Lang, the volume change is followed by the change in density it produces in a small aqueous drop suspended in a nonaqueous medium. The measurement of the density is ingeniously made by recording the position of the drop in a density gradient maintained in the nonaqueous fluid. A change in density is reflected in a vertical displacement of the drop to a new position where the densities of the drop and the surrounding medium are again equal. The magnitude of the displacement then becomes a measure of the extent of the reaction that occurred within the drop. By introducing aqueous drops of salt solutions of known densities, a simple calibration of the density gradient in the nonaqueous phase is obtained.

The density gradient is produced by half filling a tube with bromobenzene or bromobenzene-kerosene mixture having a density greater than one, and then covering this liquid with a column of similar mixture or kerosene having a density less than one. By mixing the liquids in the center where the two layers join, a density gradient is obtained. If the tube containing the liquid is held in a good thermostat, the gradient will be linear and remarkably constant for months at a time.



Obviously the method is not suited to those systems that contain or evolve substances soluble in the nonaqueous medium, *e.g.*, lipase measurements could not be made in this manner. The sensitivity of the method for a given reaction depends primarily on the magnitude of the contraction constant of the reaction. This constant is defined as the volume change occurring when one gram mole of reactant undergoes chemical change. Thus, the contraction constant for the hydrolysis of urea is 24.1, and accordingly when 60 g. of urea is hydrolyzed the volume of the reaction mixture will decrease by 24.1 ml.

The fact that the measurements can be made without affecting the reacting system in any way by the simple use of a cathetometer to determine the vertical displacements is no small advantage. For peptidase work, 0.10 to 0.15  $\mu$ l. drops of reaction mixture may be used. Linderstrøm-Lang and Lanz (115) calculated that in a ten hour reaction period, with 0.10  $\mu$ l. drops and density measurements to  $1 \times 10^{-5}$ , the hydrolysis of alanyl-glycine by one thousandth of one sea urchin egg ( $5 \times 10^{-8}$  mg. dry weight) can be detected.

The use of the gradient tube for measurements of "reduced weight" has already been mentioned.

#### IV. The Outlook

From the preceding brief survey it must be apparent that even in their present newly developing forms enzymic histo- and cytochemistry are drawing extensively on diverse analytical techniques. While the attainment of the objective of the quantitation of enzyme activities in the finest anatomical entities is still to be discerned only dimly in the distance, the progress already achieved is encouraging.

The enzyme, perhaps more than any other single biological constituent, characterizes living matter, but, of course, enzyme action is conditioned by many cellular constituents. Histo- and cytochemistry encompass all biological substances. Looking at the field as a whole, what can be expected of future developments? From knowledge of the chemistry of the structures which he delineates, the anatomist will begin to see beyond the dead end of pure morphology. In the chemical topography of tissues and cells, the pathologist will find a greater understanding of the changes inflicted by disease. From observations of the chemical changes in the cellular com-

ponents in various functional states, and under the stress of drug action, the physiologist and pharmacologist will find a greater exposition of the mechanisms which actuate the living form. In the chemical constitution of the architectural elements that comprise the matter of life, the chemist will find a more exact formulation of its molecular patterns and a clearer definition of their properties.

But, in all of this, the stiff membranes which have grown to segregate the life sciences will become more permeable, and, in the freer interchange, a body of knowledge will evolve which will have an increasing dynamic coordination and vigor.

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# ENZYME ACTIVITY IN FROZEN VEGETABLE TISSUE

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## I. Introduction

That changes occur in the flavor of most untreated vegetables during freezing storage is now common experience (59,93,122,125). These changes in flavor have been ascribed to the activity of naturally occurring enzymes not inhibited by low temperature and ice formation. Just what enzyme systems or substrates are involved is still not known in spite of the fact that about twenty years have elapsed since Haslach (47) first patented a process of preserving vegetables by freezing and since Joslyn and Cruess (57,63), Kohman (75), Barker (12), and Diehl *et al.* (36) laid the foundation for the methods used at present in commerce (122). The undesirable off-flavors produced on freezing storage of raw or underblanched vegetables are believed to be due to the accumulation of unknown rather volatile compounds. There is good evidence, however, to believe that one or more of the respiratory enzymes are involved and that the off-flavors produced are similar to those present in plant tissues undergoing "anaerobic respiration"—actually fermentation (42) or anaerobiosis—as a result of suboxidation or mechanical injury. The present technological and scientific data in this field will be critically presented more to stress the need for additional investigations in this field than to summarize the largely empirically obtained data.



## II. Evidence for the Enzymic Nature of Flavor Changes

In the early industrial development in this field it was not realized that deterioration in quality of frozen vegetables would be due to enzyme activity. Thus Haslach (47), in filing his patent in 1926, explained his application of boiling as a pretreatment to expel air and "fix" the transformed chemical constituents to prevent absorption from vegetable stalks of deleterious flavoring ingredients. Birdseye (18), in filing his patent in 1930, partially cooked spinach prior to freezing to remove a portion of its moisture content, render it pliable and compact, and partially break down its cellular structure. Tressler (119) became aware of enzymic action by noticing that all vegetables except spinach which were originally frozen by the Birdseye organization developed off-flavors. Kohman (75) had already pointed out in 1928 that cooking vegetables merely enough for serving preserves their flavor in freezing storage, since freezing inhibits but does not destroy plant enzymes, particularly respiratory enzymes. He had previously called attention, in a general way, to the fact that enzyme activity as well as microbial activity was involved in the deterioration of fruits and vegetables (74). Barker in 1930 also recommended partial cooking for the prevention of "autolysis" in peas (12). Joslyn and Cruess (63), and Joslyn (57) reported good flavor retention in vegetables scalded in steam or water for two to five minutes, whereas the raw vegetables developed off-flavors during freezing storage. These and other similar investigations merely indicate that a thermolabile system is involved in the formation of off-flavors without proving that this is a single or multiple enzyme system.

The evidence for enzyme activity was strengthened by the discovery that the production of off-flavors occurred gradually during freezing storage and that the lower the temperature at which the vegetable tissue was scalded or the shorter the period of scalding the more rapidly did these off-flavors develop (64-66). An extensive series of tests was carried out in our laboratory to determine the effect of temperature and time of blanching (scalding) on the rate at which off-flavors developed in freezing storage and the relation of this to enzyme activity. Since the nature of the enzyme-substrate systems was unknown, our first approach was to determine the relation between readily determinable plant enzymes and flavor

retention. Our attention was focused at first on the relations in peroxidase activities, but the earlier qualitative tests indicated that peroxidase activity as indicated by the widely used benzidine test did not parallel flavor retention (64,66). The inactivation temperature for peroxidase by this test was considerably higher and the time longer than that necessary to inactivate the more thermolabile enzymes responsible for flavor changes. In subsequent investigations, as will be discussed later, we found a difference between the thermal inactivation rate for various vegetable peroxidases when different indicators were used both for qualitative and quantitative tests. This may be due to the existence of several types of peroxidase systems.

At about the same time that our investigations were in progress, Diehl *et al.* (36) reported that the catalase activity in Alderman peas served as an index of the adequacy of scalding. Peas blanched at sufficiently high temperatures and for a time sufficient to destroy catalase remain unaltered in flavor when stored for several months at  $-6.5^{\circ}\text{C}$ . or lower. Peas showing positive catalase activity developed haylike flavors and odors with attendant changes in color.

Catalase activity and peroxidase activity have been used as criteria for adequacy of the scalding treatment only because under certain conditions their thermal death rates parallel those of the enzyme systems involved in production of off-flavors. The actual system or systems involved are not known. Barker (12) and Barker and Morris (13-15,97) refer to the process as autolysis. Tressler (119) believed that the reactions involved were both hydrolytic and oxidative in nature. Tyrosinase, catalase, and possibly proteinases were believed to be involved. The basis for assuming that proteolytic changes were involved was the difference in behavior on cooking of properly blanched and underblanched frozen peas. In cooking the former little scum and no foaming occurs whereas with the latter there is much foaming and scum formation. Mergentime and Wiegand (93) reported the occurrence of a proteolytic enzyme in filtered water extracts of peas which was similar to muscle cathepsin. This crude enzyme produced proteolysis at temperatures as low as  $-10^{\circ}$  without ice formation, although ice formation reduced the rate, the reduction being greater at  $-10^{\circ}$  than at  $-5^{\circ}$ . Since there is evidence that proteolysis does occur in unscalded frozen vegetables stored at  $-17^{\circ}$  they believe this to be important

in the deterioration in quality of such vegetables. They reported that the products of proteolysis of casein by this pea extract were malodorous and distasteful but since they used thymol, a relatively poor bactericide, it is not known to what extent bacteriological changes were involved, particularly in tests in which the pea juice-casein substrate was incubated at temperatures of 40° for 24 hours. The enzymic hydrolysis of casein into amino acids and peptides is not normally accompanied by volatile malodorous substances.

The malodorous substances produced in insufficiently scalded or raw vegetables are due largely to formation of small amounts of

TABLE I  
CHARACTERISTIC ODORS OF UNBLANCHED FROZEN VEGETABLES (59)\*

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Artichoke hearts	.....Sharp silage odor with fishy background.
Asparagus	.....Sharp silage odor reminiscent of overheated grass in stacks; butts have milder off-odor than tips or stalks.
Beans, green	.....Sweet alfalfa odor.
Beans, horse	.....Pungent sweet butyric odor.
Beans, Lima	.....Odor of oil of bitter almonds; slightly putrid.
Brussels sprouts	.....Stale cabbage odor.
Peas	.....Fruity alfalfa odor.
Spinach	.....Sharp alfalfa odor; tobacco or herring brine.
Squash	.....Sharp acetaldehydelike odor; burnt or rotten pumpkin.

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\* After storage for two to four years at -17°C.

rather volatile compounds whose nature so far is unknown. These substances are volatile with steam. The off-odor in frozen vegetable tissue is reduced in intensity on distillation and the odoriferous substances accumulate in the distillate. They are also extractable with solvents like ethyl ether, petroleum ether, or benzene. In peas they have been referred to as being similar to old hay, decaying grass, or the smell of chicken gizzard contents (34,64). Although the off-odors formed in all raw or underscalded vegetables are sufficiently similar to be termed "haylike," they differ with each vegetable ranging from the distinctly alfalfalike odor developed in green beans to the sharp acrid odor in squash, as shown in Table I. Undesirable flavor changes follow closely upon the detection of these odors which are noticeable in raw peas as early as six weeks after storage at

—5° and increase in intensity with storage period (35). In other vegetables their formation is usually slower; raw broccoli, for example, may be stored for over six months at —17° without change in odor but this is quite definite at the end of a year. These off-odors and off-flavors will form in vegetables frozen and stored in the absence as well as in the presence of oxygen and will form even when the gases in the intercellular spaces are removed by vacuumizing while submerged in water or salt solutions. Oxidation apparently is not involved.

It is now well established that plant tissues stored in atmospheres of low oxygen content will undergo abnormal respiration with the formation of alcohol and acetaldehyde and the development of off-flavors. Kohman (74–76) and Kohman and Sanborn (77) have stressed the importance of such “anaerobic respiration,” preferably called fermentation (42), in the deterioration of quality in these products as judged by ordinary senses. Kohman and Sanborn (77) particularly have shown that the bruising of succulent vegetables has a profound effect on their respiratory systems. While the carbon dioxide evolution in the bruised peas and Lima beans was almost similar to that of the unbruised controls, the oxygen consumption decreased markedly, and alcohol and acetaldehyde were formed in quantities much larger than in the unbruised vegetables. A similar decrease in oxygen consumption was found by Bonner and Wildman (19b) in spinach leaves subjected to freezing and thawing. Kohman and Sanborn (77) reported that unbruised peas and Lima beans submitted to anaerobiosis and the raw frozen vegetables developed off-flavors similar to those found in bruised vegetables and suggested that vegetable tissue subjected to freezing and thawing may be regarded as being completely bruised. The possibility that enzyme activity in frozen plant tissues occurs during freezing storage with the accumulation of by-products similar to those occurring in bruised tissues has many important consequences. Neglecting for the time being the changes in rate with change of state caused by ice formation, large changes in concentration of solutes and enzymes occur as a result of this ice formation and enzyme activity must occur in a fairly concentrated, unfrozen liquid medium. In this connection it is of interest that off-flavors similar to those found in raw frozen vegetables occur also in raw dehydrated vegetables (28). Highly osmophilic enzyme systems must be involved in such tissues.

Although the general mechanism of respiration is known, our knowledge of the intermediary metabolism of carbohydrate respiration of plants is still meager (54). The normal enzymic processes in living cells are regulated in rate and order and are coupled with

TABLE II  
EFFECT OF SCALDING CONDITIONS ON FLAVOR RETENTION AND ENZYME ACTIVITY  
IN MARTHA WASHINGTON ASPARAGUS

Scalding conditions			Enzyme activity						Flavor
Temp., °C.	Time, min.	Year	Peroxi-dase <sup>a</sup>	Peroxi-dase <sup>b</sup>	Cata-lase	Catalase, kat. l/g	Peroxidase, P.E/g.	Aldehyde, mg./kg.	
20	2	1933	4+	4+	4+	2.53	0.0055	22.0	Off
40	2	1933	4+	4+	4+	2.84	0.0056	29.8	Off
50	2	1933	4+	4+	4+	2.48	0.0053	29.0	Off
60	2	1933	4+	4+	4+	2.24	0.0049	41.4	Off
65	2	1933	4+	4+	4+	0.98	0.0016	26.0	Off
70	2	1933	4+	4+	3+	0.60	0.00092	14.1	Off
75	2	1933	4+	4+	3+	0.07	0.00060	5.2	Off
80	2	1933	4+	3+	3+	0.008	0.00052	3.1	Off
85	2	1933	4+	3+	+	0.009	0.00021	2.2	Off
90	2	1933	3+	3+	+	0	0.0000	2.6	Sl. off
95	2	1933	2+	2+	+	0	0.0000	1.3	Fair
100	2	1933	1+	+	+	0	0.0000	1.7	Fair
100	¼	1933	4+	4+	4+	0.88	0.0019	37.0	Fair
100	½	1933	4+	4+	4+	0.41	0.0011	21.1	Fair
100	1	1933	4+	3+	2+	0.026	0.00017	3.1	Fair
100	2	1933	3+	2+	+	0.00	0.0000	1.8	Fair
100	5	1933	+	—	+	...	...	...	Fair
100	1	1937	4+	4+	4+	0.181	0.0000	5.7	Fair
100	2	1937	4+	2+	3+	0.013	0.0000	...	Fair
100	3	1937	+	—	+	...	...	...	Fair
100	5	1937	+	—	—	...	...	...	Fair
100	1	1938	4+	+	2+	0.0119	0.0000	...	Fair
100	2	1938	3+	—	—	0.0066	0.0000	...	Fair
100	3	1938	+	—	—	...	...	...	Fair

<sup>a</sup> Benzidine.    <sup>b</sup> Guaiacum.

each other, either directly or through intermediates so that any given over-all reaction, such as respiration, proceeds by a series of well-defined steps. Glucose, *e.g.*, when respired in the presence of oxygen is converted to carbon dioxide and water by a series of coupled individual enzymic reactions (41,50). In the first phase

of this (formation of pyruvate from glucose), twelve separate enzymic steps and one nonenzymic step are involved, in each of which an intermediate formed as a product of the preceding reaction is used up in the succeeding one (16). In the second phase which is not so well defined, from three to over twelve enzyme systems are involved (78). Anaerobically the glucose molecule passes through twelve stable intermediary stages before being converted into alcohol and carbon dioxide and this transformation involves at least three dissociable organic coenzymes, twenty or

TABLE III

EFFECT OF SCALDING CONDITIONS ON FLAVOR RETENTION AND ENZYME ACTIVITY IN CUT KENTUCKY WONDER GREEN BEANS

Scalding conditions		Enzyme activity			Flavor	Catalase, kat f./g.	Peroxidase, P E./g.	Aldehyde, mg./kg
Temp, °C.	Time, min.	Catalase	Peroxi-dase <sup>a</sup>	Peroxi-dase <sup>b</sup>				
20	2	3+	4+	4+	Off	0.111	1.13	25.0
40	2	3+	4+	3+	Off	0.115	1.10	24.2
50	2	...	...	...	...	0.091	0.98	22.8
60	2	...	...	...	...	0.067	1.10	15.8
65	2	+	4+	3+	Off	0.033	1.02	9.6
75	2	...	...	...	...	0.000	0.61	9.6
77.5	2	—	4+	3+	Off	0.0000	0.47	8.8
80	2	—	4+	3+	Off	...	0.32	5.2
82.5	2	—	4+	3+	Fair	...	0.25	3.5
85	2	—	4+	+	Fair	...	0.20	3.5
87.5	2	—	4+	—	Fair	...	0.018	3.5
90	2	—	3+	—	Fair	...	...	3.5
100	2	—	3+	—	Fair	...	...	3.5

<sup>a</sup> Benzidine.

<sup>b</sup> Guaiacum.

more enzyme proteins, and some bivalent metals (manganese, magnesium) (94). These individual enzymic reactions are controlled in the intact cell in such a manner that the intermediate products do not accumulate, but when the cells are damaged by freezing or mechanical injury these intermediates may appear. Furthermore enzymes like catalase, peroxidase, and phenolases, whose role in the over-all reaction is not clear, may become activated and produce abnormal changes.

The temperature coefficients of many of the intermediary enzymic reactions are not known but from the available knowledge they

should differ significantly so that lowering of temperature alone should produce an imbalance which would lead to accumulation of intermediates. Changes in concentration and enzyme activity of

TABLE IV  
EFFECT OF CONDITION OF SCALDING ON FLAVOR RETENTION AND ENZYME ACTIVITY  
IN HENDERSON BUSH LIMA BEANS

Scalding conditions		Flavor	Catalase, kat. f./g.	Peroxidase, P.E./g.	Alde- hyde, mg./kg.
Temp., °C.	Time, min.				
71	5	Off	0.067	0.0022	10
	10	Off	0.053	0.0010	2.2
	15	Sl. off	0.059	0.0012	0.9
77	5	Off	0.035	0.0012	1.8
	10	Sl. off	0.044	0.00084	2.6
	15	Fair	0.039	0.00066	0.9
82	2	Off	0.051	0.00097	6.1
	5	Off	0.025	0.00060	3.0
	10	Fair	0.035	0.00061	1.3
	15	Fair	0.011	0.00023	0.0
85	2	Off	0.055	0.00053	7.9
	5	Sl. off	0.044	0.00041	0.0
88	2	Off	0.046	0.00049	5.3
	5	Sl. off	0.034	0.00040	1.3
	10	Fair	0.0089	...	0.4
	15	Fair	0.0000	...	0.0
91	2	Off	0.037	0.00042	6.1
	5	Fair	0.023	0.00020	1.7
93	2	Off	0.015	0.00027	9.2
	5	Fair	0.0036	...	4.8
	7	Good	0.013	...	4.4
	10	Good	0.000	...	4.4
100	1	Off	0.028	0.00022	8.8
	2	Off	0.019	...	4.8
	3	Off	0.000	...	3.5
	4	Fair	0	0.00020	4.4
	5	Good	0	...	0.9
Unblanched			0.60	0.0073	175

the catalysts and of the enzyme-substrate compound as well as changes in permeability, as discussed by Brooks (21), would be involved also. The nature and extent of these changes is at present unknown. It is well established that acetaldehyde is formed in plants both under anaerobic and aerobic conditions. Under anaerobic conditions it is most likely formed from pyruvic acid by decarboxylation and may then be reduced to alcohol or further transformed. Kohman and Sanborn (77) identified acetaldehyde in bruised peas by dimethylcyclohexanedione derivatives and estimated the aldehyde and alcohol formed by bruising or anaerobiosis. Neuberg and Gottschalk (97a) and Bodnar *et al.* (19a) definitely proved that enzymes in pea tissues could form acetaldehyde and ethyl alcohol under anaerobic conditions. Arighi, Joslyn, and Marsh (3) found that volatile aldehydes accumulated through enzyme activity in tissues of frozen peas. The aldehyde content of peas was found to be a good indication of quality since the quantity present decreased with decrease in catalase activity and paralleled flavor retention, being least in peas which retained flavor and highest in off-flavored samples. In spinach, however, the volatile aldehyde content could not be used as an index of flavor quality, since it remained constant irrespective of treatment. Aldehyde accumulation was observed also in artichoke hearts (62), green snap beans (17), asparagus (60), and in Lima beans, Brussels sprouts, and squash (61). As in the case of spinach, aldehyde accumulation did not occur in broccoli (23).

The relative extent of accumulation of aldehydes, expressed as acetaldehyde in relation to enzyme activity, is shown in Tables II to VI. There is a very marked decrease in aldehyde content with decrease in catalase and peroxidase activity in asparagus, green beans, Lima beans, and peas, but no decrease in spinach. In experimentally packed samples the aldehyde content of asparagus dropped from about 30 p.p.m. for raw or underblanched samples to less than 2 p.p.m. in properly blanched lots, for green snap beans from 25 to 3.5, for Lima beans from 10 to less than 1, for peas from 53 to about 7, but remained relatively constant in spinach.

The volatile aldehydes which in the above studies were reported



TABLE V  
EFFECT OF SCALDING CONDITIONS ON FLAVOR RETENTION AND ENZYME ACTIVITY IN PEAS

Scalding conditions		Enzyme activity						Catalase, kat. f/g.	Peroxidase, P E/g	Aldehyde, mg./kg.
		At time of scalding		After 1 year storage						
				Catalase	Peroxidase	Catalase	Peroxidase <sup>a</sup>			
Temp. °C	Time, min	Catalase	Peroxidase	Catalase	Peroxidase	Catalase	Peroxidase <sup>a</sup>	Peroxidase <sup>b</sup>	Flavor	
None	—	4+	4+	4+	4+	4+	4+	4+	Off	0.0080 53
60	2	4+	4+	4+	4+	4+	4+	4+	Off	0.0042 17.5
71	2	2+	2+	+	+	+	+	4+	Off	0.0039 20.2
77	2	+	2+	+	+	+	+	2+	Fair	0.0026 9.7
82	2	+	+	+	+	+	+	2+	Good	0.0008 5.3
85	1	2+	2+	4+	3+	4+	4+	4+	Fair	0.0021 12.0
	2	+	+	+	3+	2+	2+	2+	Fair	0.0007 5.7
	3	+	+	+	+	2+	2+	2+	Fair	0.0005 7.0
88	2	+	+	+	+	+	+	2+	Fair	0.0004 7.0
93	2	+	±	+	+	+	+	2+	Fair	0.0005 7.5
100	½	+	+	+	3+	2+	2+	2+	Good	0.0017 7.9
	1	+	+	+	+	+	+	2+	Good	0.0010 9.2
	2	+	±	+	+	+	+	+	Fair	...
	3	+	±	+	+	+	+	+	Fair	...

<sup>a</sup> Benzidine.<sup>b</sup> Guaiacum.

as acetaldehyde were examined recently by David (29), who identified acetaldehyde in fresh and frozen peas by means of the 2,4-dinitrophenylhydrazine as the chief aldehyde constituent but also showed that acetyl methyl carbinol and diacetyl were formed as well as traces of other as yet unidentified aldehydes. J. David (29) demonstrated, in addition, that ethanol occurred in peas and increased in amount during anaerobiosis. Three lots of freshly shelled peas were found to contain 1.06, 1.36 and 1.85 milligram per cent of total aldehyde and 29.4, 41.8, and 35.4 milligram per cent of ethanol, respectively. After storage under anaerobic conditions

TABLE VI  
EFFECT OF CONDITIONS OF SCALDING ON FLAVOR RETENTION  
AND ENZYME INACTIVATION IN SPINACH

Scalding temp., °C. <sup>a</sup>	Catalase, kat. l./g	Peroxidase, P E /g	Flavor	Aldehyde, mg./kg.
20	2.18	0.0044	Off	17.6
40	3.71	0.0061	Off	17.6
50	3.34	0.0063	Off	17.6
60	2.96	0.0043	Off	17.6
65	0.99	0.0032	Off	17.6
70	0.124	0.0020	Off	17.6
72.5	0	...	Off	17.6
75	0.000	0.00075	Off	17.6
80	...	0.00075	Off	17.6
85	...	...	Off	17.6
90	...	...	Good	17.6
95	...	...	Good	17.6
100	...	...	Good	17.6

<sup>a</sup> For two minutes.

(using sterile technique) the respective values for aldehyde content were 2.98, 2.60, and 4.10, and for ethanol, 176.0, 185.1, and 270.4. As a result of anaerobiosis the aldehyde content had doubled but the alcohol content had increased sixfold. In frozen raw and under-blanching peas there was also an accumulation of aldehyde and alcohol. The effect of scalding conditions on the accumulation of aldehyde and alcohol in peas is shown in Table VII. There is evidence of an appreciable increase in acetaldehyde content during freezing storage at  $-17^{\circ}\text{C}$ ., the increase being greater in under-blanching samples. The ethanol content of frozen peas, however, was

markedly greater, about twenty times as large as acetaldehyde content. Using similar technique Buck (23) found accumulation of alcohol in frozen raw and underblanched broccoli. The presence of carboxylase in higher plants has been established only recently (54,123); data on the distribution and activity of alcohol dehydro-

TABLE VII  
EFFECT OF SCALDING TEMPERATURE ON ALDEHYDE AND ALCOHOL  
ACCUMULATION IN PEAS

Scalding temp., °C.*	Aldehyde, mg./kg. after storage for		Alcohol after 6 years, mg. %	Alcohol Aldehyde
	1 year	6 years		
60	17.5	24.7	46.9	1.90
71	20.2	16.0	16.9	1.05
77	9.7	14.0	21.9	1.57
82	5.3	16.9	15.8	0.94
88	7.0	12.5	14.5	1.16
93	7.5	11.6	14.8	1.27
100	...	10.6	16.1	1.54

\* Two minutes.

genase are still meager (54). It would appear that alcohol dehydrogenase was active in frozen vegetable tissue, particularly peas and broccoli.

It is obvious from the foregoing discussion that enzyme activity occurs in frozen vegetable tissues but what enzymes are involved, what substrates are acted upon, and what products are formed is still unknown. It is evident, however, that the compounds responsible for off-flavor are formed from some precursor during freezing storage in a rather concentrated liquid phase. Von Loesecke (82a) suggested that the hay-like flavor which develops in dehydrated spinach (similar to that which develops in frozen spinach) may be due to coumarin. Mazé (90a) suggested that coumarin which he reported to occur in bean leaves may be formed from diacetyl. The relation of acetaldehyde content to intensity of off-flavor, and the occurrence of diacetyl in frozen peas lends credence to the possible conversion of diacetyl, pyruvic acid, or acetaldehyde into coumarin by a reaction similar to the following:



### III. Effect of Freezing on Enzyme Activity

It has long been recognized that low temperatures and ice formation do not inhibit although they do retard enzyme activity. The rate of enzyme action is decreased as the temperature is reduced but, because the rate of decrease is not the same for all enzyme-substrate systems, refrigeration may bring about an imbalance in the chain of stepwise enzyme reactions leading to the accumulation of intermediates. The sweetening of potatoes, pithing of grapefruit, and purpling of cauliflower are examples of the well-recognized low temperature injury in plants. Enzymes also differ in their power to survive prolonged exposure to freezing temperatures, and changes in the enzyme systems by partial destruction of the more labile enzymes such as the dehydrogenases may alter the type as well as the rate of chemical change produced. The early literature on the influence of low temperatures was summarized by Hepburn (48), who reported that a number of enzymes survived prolonged exposure to temperatures varying from about 0°C. to as low as  $-191^{\circ}$  (liquid air), either in the tissues or in solution. Sizer (109) in his recent review of the effects of temperature on enzyme kinetics again called attention to the fact that enzymes are not inactivated by storage at temperatures as low as  $-186^{\circ}$ .

The earlier investigations of the effect of freezing on enzyme activity, however, for the most part have been largely qualitative in nature. Nord and collaborators, however, during the period 1928 to 1938 (52,79,98-100) have called attention to transient increases in activity of frozen solutions of zymases, peroxidases, and tyrosinase under some conditions and decreases under others. He concluded that these effects could be explained by the fact that on exposure to freezing, particles of aqueous solutions of lyophilic biocolloids will undergo disaggregation or aggregation according to the prevailing concentration of the particles. This was readily demonstrable in differences in the speed of diffusion of frozen (at  $-17^{\circ}$  and  $-79^{\circ}$ ) and unfrozen solutions of sodium oleate, ovalbumin, and polyacrylic acid. In solutions of concentration up to 1%, there was a demonstrable disaggregation on freezing evidenced by a measurable increase in speed of diffusion, and in concentrations higher than 1.5% a measurable aggregation manifested by a decreased speed of diffusion in comparison to that of the unfrozen solutions (100). An

understanding of the colloidal readjustment and ionic shifts brought about by freezing is necessary to extend our knowledge of the effect of freezing on enzyme activity.

Similar effects were observed by Kiermeier (73), who found that catalase and lipase solutions retained their activity on freezing and were actually more active in the frozen than in the undercooled liquid state. After thawing, the enzymes were more active than before freezing. In natural products this was not always true. Repeated freezing, however, precipitates the enzymes, not only in aqueous solutions but also in extracts of potatoes. The freezing effect was strongly influenced by such effects as the rate of freezing; more catalase activity was lost on freezing slowly than rapidly.

Carrick (26) found that extreme freezing of apples in which most if not all of the cells were killed markedly reduced catalase activity. Freezing for three to nine hours at an air temperature of  $-7.5^{\circ}$  resulted in increased catalase activity of the vascular tissue but after twenty hours there was a reduction in catalase activity. A varietal difference was observed between McIntosh and Baldwin apples. Kaloyereas (69) reported less catalase activity in spinach frozen at  $-65^{\circ}$  than in that frozen at  $-17^{\circ}$ .

The activity of a number of enzymes has been observed in the frozen state: invertase (65,68,72), pancreatic lipase (10,11,73), catalase (73), protease (4,10,93), pectic enzymes (65), and peroxidase (106). The effect of freezing upon the enzymes and substrates acted on has been investigated in a preliminary way but our knowledge of this is still meager. Quantitative studies on enzymic changes in frozen plant or animal tissue, however, are also very meager. Inversion of sucrose in frozen fruit (65,68), loss in pectin during freezing storage (65,97), increase in total sugars in frozen potatoes (12), and proteolysis of frozen animal tissues (10,11) and of frozen peas (93) has been reported. The disorganization of protoplasm and its attendant effect on extensive enzyme and colloidal changes has been studied by plant physiologists (27,31,58)—primarily to obtain information on the mechanism of killing of plant cells by freezing—and by Luyet and collaborators (84–88)—to obtain fundamental data on the response of protoplasm to freezing conditions. While these studies are useful in advancing our knowledge of the behavior of tissue enzyme systems at low temperatures they are still incomplete.

The kinetics of enzyme activity at low temperatures has been investigated to but a limited extent. Studies of the effect of temperature on respiratory and other enzymic activity in vegetable tissue are even more limited (2,19,103). Balls and Tucker (11), Joslyn and Sherrill (68), Kertesz (71,72), Lineweaver (80), Mergentime and Wiegand (93), Saatchan (106), and Sizer (108-110) have reported data on several enzyme systems. In general these data indicate that, although freezing does not inhibit enzyme activity and even though enzyme action continues to occur in the frozen state, ice formation has a marked retarding effect. Lineweaver (80), and Sizer and Josephson (110) recently called attention to the fact that for the few cases investigated the velocity of enzyme reaction is faster in the supercooled state than in the frozen state at the same temperature, *i.e.*, that the velocity greatly decreases when the change of state occurs. This decrease must be due to increase in concentration of substrate and to other changes which take place in the physical and colloidal properties of a system when it passes from liquid to a solid state. This conclusion was confirmed by Mergentime and Wiegand (93) for crude pea proteinase, and by Kertesz (72) for invertase, but was not confirmed by Kiermeier (73) for the catalase or lipase preparations he studied.

The limited study that has been made of the effect of storage temperature on rate of production of off-flavors in frozen vegetables indicates that this rate decreases with decrease in temperature. At  $-17^{\circ}$ , according to Tressler (121), enzyme action is still sufficiently rapid to change the flavor of unblanched vegetables in a few weeks; he states: "If, however, the temperature is lowered to  $-45^{\circ}\text{C}$ ., the rate of enzyme action is so greatly reduced that most unblanched vegetables may be kept for six months or longer without the development of off-flavors."

#### IV. Scalding as a Pretreatment for Flavor Retention

Since the usual cold storage temperatures are seldom much lower than  $-22^{\circ}\text{C}$ ., it is essential that all the vegetables be treated to inactivate the enzymes concerned in off-flavor formation. To accomplish this, the early investigators introduced the practice of scalding in steam or hot water, followed by subsequent cooling in water or air. The practice, now well established in the industry as

as essential step in the preparation of vegetables for preservation by freezing (122), was introduced before there was any knowledge of the nature of the enzyme involved, the substrates acted upon, or the type of changes brought about in the product (see the reviews of early investigators: 3,59,66).

In establishing the required scalding period, vegetables were heated for various periods of time at different temperatures, stored for various periods of time at temperatures of about  $-17^{\circ}$ , and periodically examined. The degree of flavor retention in these vegetables was compared to the activity of enzymes that could be readily determined or that were suspected of being concerned in the formation of undesirable flavors. As a result of such tests an empirical correlation was observed between the activity of certain test enzymes used as indicators and flavor retention. The test enzymes used were catalase and peroxidase. In general, if the scalding treatment is not sufficient to inactivate catalase and peroxidase, the frozen vegetables will develop off-flavors; and, conversely, commercially processed samples which are off in flavor will contain active catalase and peroxidase enzymes (22,39,105,122).

The problems involved in destruction of enzymic activity by heat have been summarized by Balls (5,6), who stressed the difficulty of complete destruction, the possibility that the products of decomposition by heat might exert slight catalytic action, and the possible regeneration of activity after destruction. This regeneration is well recognized and has long been known to occur with proteolytic enzymes and peroxidase (45,49,107). There is some evidence that both catalase and peroxidase are regenerated during freezing storage; this is particularly true when qualitative tests are used. It has been observed on numerous cases in our laboratory that vegetables which give no immediate reaction with hydrogen peroxide or with hydrogen peroxide plus guaiacum or guaiacol after scalding and cooling will give a slight test, particularly for catalase, after freezing storage. This was particularly true in vegetables scalded just barely sufficiently. Diehl *et al.* (35) reported that the catalase in peas inactivated for the moment by heat may be active in scalded and cooled peas one-half hour afterward. Woodroof *et al.* (125) have found this to be true for peroxidase activity in snap beans and squash. Quantitative differences, however, were small even after four years'

storage at  $-17^{\circ}$ . There is little indication that such regeneration contributes to the formation of off-odors and flavors.

The scalding procedure is influenced by factors that determine heat penetration, such as size and shape of individual vegetable or vegetable parts, temperature of the vegetable tissue and its heat conductivity, the temperature of the scalding medium, the relative motion of the medium and vegetables, and the nature of this medium. Several of these heat transfer determinants have been evaluated. Biological factors such as effect of variety, maturity, and growing conditions also influence rate of heat inactivation of the particular enzyme systems used as test enzymes. The slowness of heat penetration, except into small pieces of vegetables (such as individual peas), leads to variability of heat treatment so that the surface layers are subjected to more intense heating than the interior.

The distribution of the enzyme systems both as to type and concentration also is known to vary in a particular vegetable. As a rule peroxidase activity is most intense in those portions of the tissue where respiratory and other metabolic activity are greatest, and the thermal rate of inactivation varies in different plant structures. Thus Mergentime (92) found that peroxidase reaction (toward guaiacum) of baby Lima beans is quite strong in the skin but weak in the cotyledon. Joslyn (59) has observed that the rate of inactivation of peroxidases in the skins of peas and beans is much slower than in the cotyledons. In corn the peroxidase activity of the meat of the kernel is readily destroyed by scalding, but in the skin, particularly near the base where it is attached to the cob, the peroxidase is quite heat resistant (92). Mergentime found the guaiacum-blueing property of the corn cob tissue to be so strong and so heat stable that it was possible to use it to detect pieces of cob in an adequately scalded cut corn pack. Bullis and Wiegand (24) reported corn cob tissue to contain more peroxidase than the kernel and to require a longer blanch time for satisfactory inactivation. Vegetable tissue damaged as a result of suboxidation owing to improper storage conditions gives a particularly intense peroxidase reaction in the localized damaged tissue (102). This is particularly true of internally damaged tissue of potatoes.

The biological factors influencing the distribution of a particular enzyme system in the plant tissue, the occurrence of heat-stable



catalytic systems that might affect the test used, or of other factors that might affect the thermal rate of destruction are not known. An indication of the magnitude of the effect is shown in Table VIII, where a comparison is made between the time required to inactivate peroxidase in the tissue when the vegetables were heated in water at the temperatures noted, and that required to inactivate peroxidase in juice obtained from the same vegetables after freezing storage for

TABLE VIII  
THERMAL DEATH RATE OF "GUALACUM-PEROXIDASE" IN VEGETABLE TISSUES  
AND VEGETABLE JUICES (59)

Vegetable	Minutes to inactivate peroxidase in juice		Minutes to inactivate peroxidase in tissue		Minutes to retain flavor		Heat penetration (min to reach 82°C.)
	82°C.	100°C.	82°C.	100°C.	82°C.	100°C.	
Artichokes	2	1	—	7 <sup>c</sup>	—	7 <sup>c</sup>	3-5
Asparagus	2	1	4	2	—	2-3	—
Brussels sprouts	>6	4	>10, (88)	> 4, < 5	—	5-6	3-4
Cauliflower	>6	2(?)	> 9	> 3	—	3-4	—
Horse beans	2	1	> 2	2	88-2	2	—
Lima beans							
Henderson Bush	3	1	2	1	10	5	—
Peas	3(?)	1(?)	> 3	> 2	2	0.5-1	—
Spinach	>6	4(?)	6	3	—	3	—
Squash							
Italian green <sup>a</sup>	>6	>5	—	> 7, <10	—	10	6-20 <sup>d</sup>
Italian yellow	>6	>5	—	>10	—	10	8-19 <sup>e</sup>
Summer <sup>a, b</sup>	>6	1	—	>12	—	10	5-12 <sup>f</sup>
String beans	>6	3	> 5	2	88-4	2-3	—

<sup>a</sup> Large; cut in half.

<sup>b</sup> Medium; cut in quarters.

<sup>c</sup> 0.5% citric acid.

<sup>d</sup> 1.25 to 2.25 inch diameter.

<sup>e</sup> 1.5 to 2 inch diameter.

<sup>f</sup> 1 to 1.5 inch diameter.

several years, then grinding and expressing by hand through four layers of cheesecloth.

Three milliliter samples of the juice brought to room temperature were pipetted into  $\frac{1}{2} \times 4$  inch thin-walled test tubes and heated in an agitated water bath for the times specified. Less than one minute was required to bring the juice to both temperatures. In vegetables of sufficiently small size so that heat transfer factors were not

limiting (*e.g.*, Lima beans, peas, cut green beans) the thermal inactivation rate for the tissue was significantly faster than in the juice.

Obviously the scalding and subsequent chilling operations should be conducted under conditions such that the enzymes responsible for spoilage during freezing storage should be inactivated with the minimum effect on flavor and texture. Excessive leaching of soluble solids, which is particularly severe in the later stages of scalding and in chilling, should be avoided (51,91). The temperature of the scalding medium and the period of exposure will vary with the factors determining the heat transfer and biological factors, and failure to take this into consideration has resulted in considerable confusion in the technological literature in this field. In general the time of scalding is longer, the lower the temperature. For peas, two to three minutes at 82° is equivalent to three-fourths to one minute at 100°, but for many varieties of peas the longer scalding at the lower temperature yields a product of better texture and flavor. For cut green beans five minutes at 88° is equivalent to two minutes at 100° in water, and the former is often preferable.

## V. Catalase Activity as an Index of Scalding Efficiency

The thermal inactivation of catalase in some vegetable tissues occurs at a rate closely parallel to that of the enzymes concerned in the development of off-flavors, and where this is true catalase activity may be used as a criterion of the adequacy of scalding. The first correlation of this was reported by Diehl, Dingle, and Berry (36), who found that Alderman peas scalded at various temperatures for times long enough to inactivate catalase (qualitatively) did not form undesirable odors and did not change in color when stored at 20°F. for four months. Tressler (120) concluded that catalase may be used as an index of the activity of other enzymes in vegetables being prepared for freezing preservation. A blanching period sufficient to destroy catalase was believed to insure the destruction of other deteriorative enzymes.

On the basis of these and subsequent investigations the evolution of oxygen from hydrogen peroxide was suggested as a general qualitative test to determine whether the scalding inactivated the "respiratory enzymes" sufficiently for flavor retention during freezing storage. Tressler (119) suggested catalase as a test enzyme in

1932, and, in the first edition (1936) of his book (122), presented a method for semiquantitative determination. Diehl, Wiegand, and Berry (37) described the qualitative method as they used it in 1939, and Mergentime presented it in more detail (92). Subsequently Diehl (32) reported the hydrogen peroxide test reliable for peas and snap and Lima beans, but less useful for corn. In 1940, he reported (33): "As in the case of cut corn the qualitative test for catalase activity as an index of scalding in asparagus is unreliable." Mergentime (92) reported success in using catalase as a test enzyme in scalding peas, snap beans, Lima beans, spinach, asparagus, and corn on the cob, but preferred peroxidase for broccoli, cauliflower, and cut corn. Campbell (25) reported that the qualitative catalase test on scalded cut corn was not reliable as an index of scalding efficiency, since strongly positive tests for catalase were obtained in corn scalded far in excess of the time required for maintenance of quality during freezing storage. The guaiacum peroxidase test, however, was useful.

Our present knowledge of the chemistry of catalase and its action is summarized by Sumner (113). Catalase is rapidly destroyed by hydrogen peroxide, particularly in pure or purified preparations, and for this reason the temperature optimum for the reaction is 0–10°C. Catalase activity can be measured by determining the amount of hydrogen peroxide remaining after catalase has acted for definite time intervals at 0° or the amount of oxygen evolved. Various techniques and different apparatus are available to do this (118). The optimum conditions for the quantitative determination of catalase were fixed by Euler and Josephson in 1927 (40) as pH 6.8, 0°, and 0.01 *N* hydrogen peroxide. Balls and Hale (7), in the iodometric method they developed for the estimation of catalase in agricultural products, use a reaction mixture at pH 7.0, 0°, and 0.004 *N* in hydrogen peroxide. In our investigations it was found unnecessary to add liver extract, as suggested by them, since catalase inhibitors were not found in vegetables tested (3,17,60–62).

In the Lineweaver and Morris modification (81,96) liver extract is not used. A 50 g. sample is comminuted in a Waring blender for three minutes with about 1 g. calcium carbonate, and sufficient water to make 200 ml.; larger solid particles are removed by filtration through a gauze-backed cotton milk filter. To 10 ml. or less of this extract is added water to give a volume of 43 ml. and 5 ml. of

a 0.1 *M* phosphate buffer at pH 7, containing 20 g. dextrose per 100 ml. After mixing, 2 ml. of 0.01 *N* hydrogen peroxide is added, and the "Katalase Fahigkeit," kat. f., the first order reaction constant (log base 10), is determined at 0°C. This divided by the grams of sample per 50 ml. of reaction mixture is used as a measure of catalase content. Lineweaver points out that catalase determinations carried out at temperatures over 20° are unsatisfactory not only because of nonenzymic hydrogen peroxide decomposition but also because of the marked destruction of catalase by hydrogen peroxide.

These hydrogen peroxide concentrations, 0.017 and 0.00068%, respectively, are much lower than the concentrations commonly used for qualitative tests at room temperature—approximately 3.0%. Diehl *et al.* (37) describe the catalase test they used as follows:

"As commonly done, this test consists in breaking some of the scalded material into small pieces and placing these fragments in a clean test tube or glass. About two teaspoonfuls of active hydrogen peroxide, obtainable at any drug store, are added and continuous bubbling of gas from tissue pieces is watched. When no bubbling is observed, the scalding is deemed sufficient to insure against any undesirable odor and flavor in the product during storage. If the degree or type of gas evolution from unscalded tissues is observed by treating some raw material with hydrogen peroxide, no difficulty will be encountered in interpreting the test."

In making qualitative tests for catalase, in connection with studies on the relation of the extent of enzyme inactivation to flavor retention in frozen vegetables during the period 1933–1940, we preferred to use a freshly prepared 0.5% hydrogen peroxide solution. An amount of vegetable tissue not over 5 g., after a thorough crushing, is placed in a large test tube, covered with 5 ml. of water, after which 1 ml. of 0.5% hydrogen peroxide is added. The mixture is shaken and observed in one to two minutes. The evolution of a continuous stream of small gas bubbles at the surface of the vegetable tissue which rise to the surface is taken as evidence of catalase activity. In making the test on scalded vegetables, typical material should be selected, including some of the thickest pieces taken from positions least likely to have been exposed to heat, and the scalded vegetable should be cooled to room temperature. Since the hydrogen peroxide purchased at the drug store may have deteriorated in strength, the effectiveness of the prepared solution should be tested with fresh vegetable tissue and the same vegetable tissue boiled for ten minutes. The former should give a strong reaction, the latter

none. In making the observations, slowly evolved bubbles originating from trapped air and gas evolution occurring after two minutes should be disregarded.

The discrepancies recorded in the literature on the reliability of the catalase test as used by different investigators would have been greater if it were not for the inherent difficulty involved in determining the "end point" difference between vegetable tissue in which the catalase was almost completely inactivated and that in which complete destruction had occurred with a qualitative test. In addition, catalase in vegetable tissue is much more resistant to hydrogen peroxide than that in partly or completely purified state. Even vegetable extracts and juices behave differently from suspensions of coarse particles of tissue.

When the catalase content of vegetable tissue is determined by quantitative methods such as that of Balls and Hale, its reliability as an index of adequacy of scalding is poorer. Thus Arighi, Joslyn, and Marsh (3) found that catalase was quantitatively inactivated at a much faster rate than enzymes concerned in off-flavor production in peas and spinach. The catalase factor was 0 in peas scalded for two minutes at 65°, whereas it was necessary to heat at 88° for two minutes for flavor retention over a prolonged storage period. The same was true with snap beans (17). In asparagus, Joslyn and Bedford (60) found that catalase was more heat resistant both quantitatively and qualitatively; catalase activity persisted in commercially overblanched material. Bullis and Wiegand's data (24) and Campbell's data (25) indicate that catalase is more heat resistant than peroxidase in the corn kernel tissue.

The use of catalase activity as an index of adequate scalding, even when the less reliable qualitative tests are used, is not a generally reliable method of determining adequacy of scalding. For peas and Lima beans the minimum scalding necessary to inactivate catalase qualitatively is generally adequate to prevent off-flavor formation in freezing storage. Some variation in catalase activity in Lima beans with growing conditions has been reported; thus Smart and Brunstetter (111) found catalase in Maryland-grown Lima beans to be more heat resistant than that found by Tressler for New York-grown beans. They recommended a scalding period longer than that necessary to inactivate catalase at 100°,

and less than that necessary to inactivate catalase at 88°. Similarly for spinach and kale they found that blanching at times not quite long enough to destroy catalase yielded products of best quality (112). For corn and asparagus, catalase inactivation is not necessary for flavor retention.

TABLE IX  
EFFECT OF SCALDING CONDITIONS ON COLOR RETENTION AND ENZYME ACTIVITY  
IN ARTICHOKE HEARTS

Scalding conditions			Enzyme activity				
Medium	Temp., °C.	Time, min	Catalase	Peroxi-dase	Flavor	Color	Catalase, kat f/g
Water	100	3	+	+	Off	Off	0.058
		4	+	+	Off	Off	0.0046
		5	+	—	Off	Off	0.0029
		7	+	—	Fair	Fair	0.0030
		9	+	—	Fair	Fair	0.0017
0.5% citric	100	3	2+	+	Off	Off	0.0186
		4	+	+	Off	Off	0.0053
		5	—	±	Off	Off	0.0038
		7	—	—	Good	Good	0.0022
		9	—	—	Good	Fair	0.0005
0.75% citric	100	3	—	2+	Off	Off	0.0190
		4	—	2+	Off	...	0.0050
		5	—	+	Off	...	0.0039
		7	—	+	Fair	Good	0.0021
		9	—	—	Good	Fair	0.0012
0.1% citric	100	3	2+	2+	Off	Off	0.0201
		4	+	2+	Off	Off	0.0028
		5	—	2+	Off	Off	0.0022
		7	—	—	Good	Fair	0.0016
		9	—	—	Good	Fair	0.0030
None	None	...	...	...	Off	Off	0.635

Typical data on asparagus, artichoke hearts, green beans, Lima beans, cauliflower, peas, Brussels sprouts, squash, and spinach are summarized in Tables II–VI and IX–XII (61). In these vegetables the qualitative catalase test does not decrease in intensity as rapidly as quantitative data indicate. Sampling errors, differences in degree of extraction of enzyme, and differences in concentration of reagents relative to enzyme content, as well as temperature of test, are

involved. To reduce errors due to the first two factors we have recently found it preferable to macerate large portions of vegetables

TABLE X  
EFFECT OF SCALDING CONDITIONS ON FLAVOR RETENTION AND ENZYME ACTIVITY  
IN CAULIFLOWER

Scalding conditions		Enzyme activity				Flavor	Catalase, kat. f./g.	Peroxidase, P.E./g.
		Stalk		Flower				
Temp., °C.	Time, min.	Catalase	Peroxidase	Catalase	Peroxidase			
82	3	+	4+	+	4+	Off	...	...
	5	±	4+	+	4+	Off	0.006	0.0014
	7	—	4+	+	3+	Off	...	0.0017
	9	—	4+	...	3+	Off	...	0.0013
88	3	—	3+	...	3+	Off	...	...
	5	—	3+	...	2+	Off	0.004	0.0011
	7	—	2+	...	2+	Off	...	...
	9	—	2+	...	2+	Off	...	...
100	1	4+	4+	...	3+	Sl. off	0.0078	0.0022
	2	—	3+	...	+	Very sl. off	0.0037	0.0007
	3	—	3+	...	+	Very sl. off	...	...
Raw*	...	...	...	...	...	...	0.245	0.0255

\* Unbleached.

TABLE XI  
EFFECT OF CONDITIONS OF SCALDING ON FLAVOR RETENTION AND ENZYME ACTIVITY  
IN BRUSSELS SPROUTS

Scalding conditions		Flavor	Catalase kat. f./g.	Peroxidase, P.E./g.
Temp., °C.	Time, min.			
Unscalded		Off	0.237	0.0238
87.7	6	Sl. off	0.0037	0.0010
100	2	Off	0.026	0.0168
100	4	Sl. off	0.0045	0.0042
100	6	Very sl. off	0.0035	0.0002

in a Waring blender and use this macerate in qualitative tests. Soft vegetables, like cauliflower sprouts, can be macerated without added water; for others an equal weight of water or buffer is used. Tests

on such macerates are more reliable than on small amounts of poorly crushed tissue.

The quantitative data indicate that the catalase factor, kat. f. per gram, must be reduced from 2.63 to 0.0000 for asparagus, from 0.635 to 0.002 for artichoke hearts, from 0.111 to 0.0000 for green beans, from 0.60 to 0.009 for Lima beans, from 0.245 to 0.0004 for

TABLE XII  
EFFECT OF SCALDING CONDITIONS ON FLAVOR RETENTION AND ENZYME ACTIVITY  
IN SQUASH AT 100°C.

Variety	Scalding time, min.	Flavor	Catalase, kat. f/g.	Peroxidase, P.E./g.
Summer	Unblanched	Off	0.103	0.013
	3	Off	0	...
	5	Off	—	0.0017
	7	Sl. off	—	0.0011
Green Italian	Unblanched	Off	0.266	0.0143
	3	Sl. off	—	...
	5	Sl. off	—	0.0021
	7	Very sl. off	...	0.0002
Yellow crookneck	Unblanched	Off	0.266	0.0190
	3	Sl. off	0.025	0.0040
	5	Sl. off	—	0.0015
	7	Very sl. off	—	...
	10	Fair	...	...

cauliflower, from 2.46 to 0.004 for peas, from 0.237 to 0.0035 for Brussels sprouts, from 0.266 to 0.000 for squash, and from 2.18 to 0.000 for spinach. Even then the asparagus, artichoke hearts, green beans, cauliflower, and spinach are not adequately scalded.

## VI. Peroxidase Activity as an Index of Scalding Efficiency

In investigations of Joslyn *et al.* (1933–1940) peroxidase activity was found to parallel enzymes in off-flavor formation more closely than catalase, but this correlation varied markedly with the nature of the substrate used for detecting peroxidase activity. Not only do the relative activities of the peroxidases of a particular vegetable tissue vary with the indicator used, but the apparent rate of thermal inactivation of the vegetable peroxidases *in situ* also varies with



the indicator. Consequently it is necessary to select the proper indicator for the particular vegetable tested. In the early investigations before this principle was established, the use of benzidine as an indicator for peroxidase activity in peas, string beans, and spinach led at first to the tentative conclusion that: "Peroxidase may not be the chief causative agent involved in deterioration of unblanched or underblanched vegetables" (64).

Peroxidases are widely distributed in plants and occur also in animal tissue. The doubt that existed concerning the occurrence of peroxidases in animal tissues, containing hemoglobin, cytochromes, and other substances, which themselves give tests for peroxidases, was dispelled by the separation and isolation of peroxidase from the leucocytes by Agner (1). To date four peroxidases have been isolated in pure crystalline form and their structures determined, namely, horse-radish peroxidase, verdoperoxidase, milk peroxidase, and cytochrome *c* peroxidase (43). The latter is unique in that it catalyzes the oxidation by hydrogen peroxide of ferrocytochrome *c* only. The other three peroxidases, in the presence of hydrogen peroxide, catalyze the oxidation of a great variety of substrates including aromatic amines, phenols, diamines, ascorbic acid, ferrocytochrome *c*, iodides to iodine, etc. Horse-radish peroxidase was used for a long time as a typical plant peroxidase but data are accumulating that plant peroxidases may differ from each other in their affinity for hydrogen peroxide (114), heat stability, and specificity with regard to reducing substances (107), etc.

A large number of indicators have been proposed for testing for peroxidases. Kastle (70) listed some forty compounds all of which have the property of being converted into readily observable, intensely colored compounds on oxidation by hydrogen peroxide in the presence of peroxidase. These indicators differ in their specificity, ease of oxidation, and other properties. In the early investigations, the action of peroxidases was investigated almost entirely with these chromogenic substances and the measurement of enzyme activity depended upon the formation of colored products. The earlier colorimetric methods of peroxidase assay were not very satisfactory from a quantitative or even a qualitative point of view. The rate of pigmentation during enzymic action is not necessarily proportional to rate of oxidation, for pigment development is markedly influenced as to degree and color by factors such as *pH*, presence of

reducing agents such as ascorbic acid, which may retard color development for some time, and other factors. The chemistry of the oxidation products produced is quite complicated, for, although the first stage in the oxidation, *e.g.*, quinone formation from hydroxybenzenes, is fairly simple, the final colored product is quite complex, for example, purpurogallin, a condensation product from pyrogallol, quinhydrone and higher oxidation products from hydroquinone, and the salt of the condensation product between diphenol-quinonediazine and unaltered benzidine, which forms as a final blue product soluble with difficulty in benzidine (45). Balls and Hale (8) used as a measure of peroxidase activity the volumetric determination of residual hydrogen peroxide and were able to extend our knowledge concerning some phases of horse-radish peroxidase.

The chromogenic indicators have been widely used until very recently in investigating peroxidase activity. Under controlled conditions they are reliable both for qualitative and quantitative measurements. One great difficulty with these peroxidase reagents is that they are all oxidized to some extent by hydrogen peroxide alone or when thermostable metallic catalysts (iron and copper salts chiefly) are present. Benzidine is least reliable in this respect, but guaiacol is not without fault, particularly in presence of certain iron porphyrin complexes. Brandrup (20), Jayle (56), Moog *et al.* (95), and Suzuki (115), among others, have stressed the catalytic effects of iron, copper, chromium, and manganese salts on peroxidase indicators. Madsen *et al.* (89) reported that an unusually long period of scalding was required to give a benzidine-negative reaction for peroxidase in beets and found 1 p.p.m. of cupric ion and 10 p.p.m. of ferric ion to give a positive benzidine reaction with hydrogen peroxide under the same conditions as the peroxidase test. Certain heat-stable compounds occur in localized areas in vegetables that act as such catalysts. Consequently the indiscriminate application of peroxidase reagents in testing for adequacy of scalding is unwise, and the results of such tests should be interpreted with caution (102).

To standardize the various possible indicators, extracts of unblanched, properly blanched, and overblanched vegetables were prepared by blending 100 g. of ground frozen vegetables stored from three to seven years at  $-17^{\circ}$  with 100 g. of washed quartz sand and 1 liter of 0.2 *M* phosphate buffer at pH 7. The extracted tissues were pressed out and the extract was allowed to settle; then 9 ml. portions

TABLE XIII  
COMPARATIVE SPECIFICITY OF VEGETABLE PEROXIDASE SYSTEMS

Substrate	Apples	para- guai- col	Arti- choke	Beans <sup>d</sup>			Brus- sels sprouts	Cauli- flower	Horse- radish	Pease	Squash <sup>b</sup>			Spin- ach	Reagent	Coloration
				1	2	3					1	2	3			
Compounds with a free OH																
<i>o</i> -Cresol	-	-	-	+	+	-	+	+	=	3+	+	+	+	+	Colorless	Antique brown
<i>m</i> -Cresol	-	-	-	+	+	-	+	+	-	-	-	-	-	-	Colorless	Light mineral gray
<i>p</i> -Cresol	-	-	=	-	-	-	-	-	-	-	-	-	-	-	Very light yellow	Light mineral gray
Guaiacol	-	+	-	+	+	+	5+	+	4+	5+	5+	5+	5+	5+	Colorless	Terra cotta
Hydroquinone	=	-	+	+	+	+	+	+	+	=	+	+	+	+	Slight yellow	Colonial buff
Hydroxy-methyl ether	-	-	-	+	+	+	+	+	+	+	+	+	+	+	Colorless	Light mineral gray
Phenol	-	-	-	5+	5+	+	4+	5+	5+	+	4+	5+	5+	+	Colorless	Light mineral gray
<i>o</i> -Naphthol	-	-	=	+	+	+	+	+	-	+	+	+	+	+	Colorless	Light pink cinnamon
Vanillin	-	+	+	+	+	+	+	+	+	+	+	+	+	+	Colorless	
Compounds with a free NH <sub>2</sub>																
Aniline	-	-	-	+	+	-	+	+	-	+	+	+	+	-	Colorless	Light mineral gray
<i>o</i> -Toluidine	-	-	-	+	+	-	+	+	-	+	+	+	+	+	Colorless	Light mineral gray
<i>m</i> -Toluidine	-	-	-	+	+	-	+	+	-	+	+	+	+	=	Very light amber	Light mineral gray
<i>p</i> -Toluidine	-	+	-	+	+	-	+	+	+	+	+	+	+	+	Colorless	Natal yellow
Compounds with 2 or more OH																
Adrenaline	-	+	+	+	+	+	+	+	-	+	+	+	+	+	Colorless	Buckthorne brown
Catechol	+	+	+	5+	5+	+	5+	+	5+	5+	5+	5+	5+	5+	Colorless	Natal brown
Chlorogenic acid	-	+	+	+	+	+	+	+	+	+	+	+	+	+	Colorless	Old gold
Gallie acid	-	5+	5+	5+	5+	+	5+	+	+	+	4+	5+	5+	5+	Very light yellow	Roman green
Guaiacum	-	+	+	+	+	+	+	+	+	+	+	+	+	+	Antique brown	American green
Guaiacum	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Pyrite yellow	American green
Hydroquinone	+	+	+	4+	+	+	5+	4+	4+	4+	4+	4+	4+	4+	Colorless	Garnet brown
Hydroxyhydroquinone	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Orange buff	Tiger brown

Substrate	Apple <sup>a</sup>	As- para- guat <sup>b</sup>	Arti- choke <sup>c</sup>	Beans <sup>d</sup>			Brus- sels	Cauli- flower	Horse- radish	Peas <sup>e</sup>	Squash <sup>f</sup>			Spin- ach	Coloration	
				1	2	3					1	2	3		Reagent	Test solution
Naphthoresorcinol	+	+++	+	+++	++	++	++	+	+++	++	++	++	++	+++	Orange citrine	Flame scarlet
Phloroglucinol	-	5+	-	5+	-	5+	-	+	+	5+	-	+	+	5+	Colorless	Light mineral gray
Pyrogallol	+	+	+	++	++	++	++	++	++	++	++	++	++	++	Colorless	Argus brown
Quercetin	+	+	+	++	++	++	++	++	++	++	++	++	++	++	Maple red	Olive lake
Quercitrin	-	-	-	++	++	++	++	-	++	++	++	++	++	++	Mars orange	Olive lake
Rosercinol	-	-	-	++	++	++	++	+	++	++	++	++	++	++	Colorless	Honey yellow
Tannic acid	-	++	+	++	++	++	++	++	++	++	++	++	++	++	Buff yellow	Dark olive buff
Tannin (coco)	+	++	+	++	++	++	++	++	++	++	++	++	++	++	Noble red	Terra cotta
Compounds with 2 NH <sub>2</sub> groups																
Benzidine	+	+	=	+	+	++	+	-	+	+	++	++	++	++	Colorless	Light drab
p-Aminodimethyl- aniline	5+	5+	+	5+	5+	5+	5+	5+	5+	5+	5+	5+	5+	5+	Carmine	Blue green black
o-Phenylenedi- amine	+	+	=	5+	4+	5+	5+	+	5+	++	++	5+	5+	5+	Xanthine orange	Orange citrine
m-Phenylenedi- amine	-	=	=	++	++	++	4+	-	++	+	+	+	+	+	Colorless	Buff citrine
p-Phenylenedi- amine	5+	5+	-	5+	5+	5+	5+	5+	5+	5+	5+	5+	5+	5+	Light maroon	Blackish purple
Compounds with an OH and an NH <sub>2</sub>																
o-Aminophenol	+	++	=	5+	++	5+	4+	+	5+	++	++	5+	5+	4+	Zinc orange	Kaiser brown
m-Aminophenol	-	+	+	4+	+	++	+	-	5+	+	4+	++	++	+	Slight yellow	Old gold
p-Aminophenol	5+	5+	+	5+	5+	5+	5+	++	5+	5+	++	5+	5+	5+	Morocco red	Natal brown
2-Amino-4-nitro- phenol	5+	4+	5+	5+	5+	5+	5+	5+	5+	4+	5+	5+	5+	5+	Mahogany red	Morocco red
5-Aminosalicylic acid	++	5+	=	4+	4+	5+	5+	++	5+	5+	5+	5+	5+	5+	Colorless	Anthracene purple
Nedi reagent	...	-	5+	...	...	...	...	...	...	+	...	...	...	...	Colorless	Livid purple

<sup>a</sup> Gravenstein, <sup>b</sup> Martha Washington, <sup>c</sup> Globe, hearts.<sup>d</sup> 1 = Kentucky Wonder, 2 = Henderson Bush Lima, 3 = horse flat beans.<sup>e</sup> Laxton Progress.<sup>f</sup> 1 = green Italian, 2 = yellow Italian, 3 = summer.

of the supernatant liquid were dispensed into test tubes, 0.5 ml. of 2% hydrogen peroxide added, followed by 0.5 ml. of 1% alcohol

TABLE XIV  
SPECIFICITY OF HORSE-RADISH PEROXIDASE

Substrate	Hydrogen peroxide utilization <sup>a</sup>	Color indicator
Compounds with a free OH		
<i>o</i> -Cresol	+	+
<i>m</i> -Cresol	...	0
<i>p</i> -Cresol	+	0
Ferulic acid	+	...
Guaiacol	+	+
Guaiacol sulfonic acid	+	...
Hydroquinone monomethyl ether	+	+
Phenol	...	+
$\alpha$ -Naphthol	0	+
Vanillin	+	+
Compounds with 2 or more OH		
Adrenaline	...	...
Catechol	+	+
Chlorogenic acid	...	+
Gallic acid	+	+
Hydroquinone	+	+
Hydroxyhydroquinone	...	+
Naphthoresorcinol	...	+
Phloroglucinol	0	+
Protocatechuic acid	+	...
Pyrogallol	+	+
Purpurogallin	+	...
Resorcinol	0	+
Tannic acid	0	+
Compounds with 2 NH <sub>2</sub>		
Benzidine	...	+
<i>o</i> -Phenylenediamine	+	+
<i>m</i> -Phenylenediamine	0	+
<i>p</i> -Phenylenediamine	+	+
<i>p</i> -Aminodimethylaniline	...	+
Compounds with an NH <sub>2</sub> and an OH		
<i>o</i> -Aminophenol	+	+
<i>m</i> -Aminophenol	0	+
<i>p</i> -Aminophenol	+	+
2-Amino-4-nitrophenol	+	+
5-Aminosalicylic acid	+	+

<sup>a</sup> After Balls and Hale (9).

TABLE XV  
COMPARISON OF PEROXIDASE INDICATORS IN UNBLANCHED, BLANCHED, AND  
OVERBLANCHED TISSUES

Reagents	Asparagus			Peas			Spinach		
	U	100, 2*	100, 5	U	82, 2	100, 2	U	100, 2	100, 3
Compounds with an OH									
<i>o</i> -Cresol	—	—	—	2+	—	—	—	—	—
<i>m</i> -Cresol	—	—	—	±	—	—	—	—	—
<i>p</i> -Cresol	—	—	—	—	—	—	—	—	—
Guaiacol	+	—	—	+	+	—	5+	—	—
Hydroquinone	—	—	—	—	—	—	—	—	—
monomethyl ether	—	—	—	+	±	—	+	—	—
Phenol	—	—	—	+	—	—	2+	—	—
$\alpha$ -Naphthol	+	—	—	+	+	—	2+	+	—
Vanillin	+	+	+	+	±	+	+	+	+
Compounds with 1 NH <sub>2</sub>									
Aniline	—	—	—	±	—	—	—	—	—
<i>o</i> -Toluidine	—	—	—	—	—	—	—	—	—
<i>m</i> -Toluidine	—	—	—	±	—	—	±	—	—
<i>p</i> -Toluidine	+	—	—	+	—	—	2+	—	—
Compounds with 2 OH									
Adrenaline	2+	—	—	2+	±	—	+	—	—
Catechol	+	+	—	5+	3+	+	5+	—	—
Chlorogenic acid	+	+	±	+	±	—	+	—	—
Gallie acid	5+	2+	—	5+	2+	±	5+	—	—
Guaiacum	2+	—	—	3+	+	±	2+	—	—
Guaiacum	3+	+	—	3+	+	—	4+	—	—
Hydroquinone	2+	2+	+	3+	3+	+	3+	±	—
Hydroxyhydro-									
quinone	3+	2+	2+	4+	2+	2+	4+	+	+
Naphthoresorcinol	3+	3+	+	3+	2+	+	3+	+	+
Phloroglucinol	—	—	—	—	—	—	—	—	—
Pyrogallol	5+	3+	+	5+	3+	3+	5+	±	±
Quercetin	+	+	+	+	+	+	+	+	+
Quercitrin	+	+	+	+	+	+	+	±	—
Resorcinol	—	—	—	+	—	—	+	—	—
Tannic acid	+	+	+	+	+	+	+	—	—
Tannin	2+	+	+	3+	+	+	2+	±	±
Compounds with 2 NH <sub>2</sub>									
Benzidine	+	±	—	+	+	—	2+	—	—
<i>p</i> -Aminodimethyl-									
aniline	5+	5+	5+	5+	3+	2+	5+	5+	5+
<i>o</i> -Phenylenediamine	+	+	+	3+	3+	+	5+	+	+
<i>m</i> -Phenylene-									
diamine	±	—	—	+	—	—	+	—	—
<i>p</i> -Phenylenediamine	5+	5+	5+	5+	5+	2+	5+	5+	5+
Compounds with an OH and an NH <sub>2</sub>									
<i>o</i> -Aminophenol	3+	2+	+	5+	3+	+	4+	+	+
<i>m</i> -Aminophenol	+	—	—	+	—	—	+	+	—
<i>p</i> -Aminophenol	5+	5+	2+	5+	2+	+	5+	2+	+
2-Amino-4-nitro-									
phenol	4+	4+	4+	5+	4+	4+	5+	4+	4+
5-Aminosalicylic									
acid	5+	5+	—	5+	+	±	5+	±	—

\* Indicates temperature of blanching water in °C., and time of blanching in minutes. U = unblanched.

solution of some forty test reagents. Immediately after addition of the reagent the solutions were shaken and observed for intensity and stability of color. Some of the data obtained in these tests carried out in 1940 with the assistance of Dr. C. L. Bedford are shown in Tables XIII to XV. Table XIII compares the intensity of color reaction observed in various raw vegetables. In Table XIV is shown a comparison between the qualitative tests with horse-radish extracts with data obtained by Balls and Hale (9), using hydrogen peroxide utilization as a test for the ability of the compounds tested to serve as substrates. Table XV shows the comparison between the intensity of reaction in underblanched and overblanched vegetables. Only those reagents which give a strong positive reaction in unblanched tissues, and a negative reaction in tissues blanched sufficiently for flavor retention, are suitable as test reagents. It was on the basis of such tests that guaiacum was found preferable to benzidine in our early tests, and guaiacol was selected as the most useful general reagent because it was more stable than guaiacum and more specific than benzidine (28).

As in the case of catalase, quantitative determinations of peroxidase activity by a modification of the Balls and Hale method (8) have not always agreed with qualitative tests suitably standardized. In the case of string beans, Bedford and Joslyn (17) found that the peroxidase activity as measured by the rate of decomposition of hydrogen peroxide in the presence of pyrogallol is definitely more resistant to heat inactivation than catalase and is closely correlated with keeping quality. The qualitative test indicated that "benzidine peroxidase" of beans was surprisingly thermostable while the "guaiacum peroxidase" was inactivated in samples that were found quantitatively to be low in peroxidase. With asparagus the guaiacum test was found to give a good indication of sufficient enzyme inactivation for flavor retention while the benzidine test was not reliable. On the other hand, Bullis and Wiegand (24) report that the benzidine test more nearly indicates the amount of peroxidase present in corn than does the guaiac test, but give no flavor comparison. Mergentime (92) reported the guaiacum test satisfactory for peas, broccoli, and cauliflower, and Masure and Campbell (90) reported the guaiacol test adequate for cut corn, asparagus, Lima beans, snap beans, and peas.

There is little information as yet concerning the percentage inactivation of peroxidase required to retain flavor over prolonged storage. All our data indicate that complete inactivation of peroxidase (measured by hydrogen peroxide utilization in the presence of pyrogallol) is not necessary, even for storage of three to seven years at  $-17^{\circ}$ ; but most of the initial activity must be destroyed. Tressler (122) suggested 99% inactivation of both catalase and peroxidase, but gave no data. The data which we have obtained during 1933–1940, only a portion of which has been published, indicate that the percentage of inactivation need not be as high as that for all vegetables, and this is supported by the data obtained by Bullis and Wiegand (24) and Masure and Campbell (90). The available published data are summarized in Table XVI. There is a strong indication that nonenzymic chemical action occurs during prolonged storage, but the significance of this in relation to commercial practice is not known (*e.g.*, see 17).

The colorimetric test for peroxidase activity is valid only when the mixture is properly balanced with respect to enzyme content (relative weight of vegetable tissue), color base or reagent, and hydrogen peroxide concentration. The relative concentrations of reagent and hydrogen peroxide used by enzyme chemists are based largely on the original work of Willstätter and Stoll in 1918 (124), who reported the optimum conditions for purified horse-radish peroxidase to be 0.25% pyrogallol, 0.0025% hydrogen peroxide, *pH* about 6, and  $20^{\circ}\text{C}$ . Balls and Hale (8) recommend 0.25% pyrogallol, 0.0027% hydrogen peroxide, *pH* 8, and  $30^{\circ}$ . Sumner and Gjessing (114) use in their final mixture approximately 0.5% pyrogallol, 0.05% hydrogen peroxide, at *pH* 6.0. While these low concentrations of hydrogen peroxide are satisfactory for purified enzyme preparations or for low dilutions of vegetable extracts which contain relatively small amounts of reducing substances such as ascorbic acid or active catalase (pyrogallol oxidation is not affected by catalase particularly in slightly alkaline solution), higher concentrations are required with vegetable extracts or macerates high in catalase and reducing substances. Even with purified peroxidase extracts differences exist; thus Sumner and Gjessing (114) report the optimum concentration of hydrogen peroxide in their modified method to be 0.025% for turnip and horse-radish peroxidase and 0.2% for milkweed peroxidase.



Much higher concentrations of hydrogen peroxide may be used with larger quantities of vegetable tissue containing active catalase

TABLE XVI  
EXTENT OF ENZYME INACTIVATION NECESSARY FOR FLAVOR RETENTION

Vegetable	Storage period	Investigators	Per cent inactivation	
			Peroxidase	Catalase
Artichoke hearts	1 yr., -17°	Joslyn, Bedford, Marsh (1938)	100	99.5
Asparagus				
Martha Washington	4.5 yr., -17°	Joslyn, Bedford (1940)	100	96.5
	2.5 yr., -17°	Joslyn, Bedford (1940)	100	99.5
Mary Washington	4 yr., -21°	Masure, Campbell (1944)	99.8	—
Beans, green cut	4 yr., -17°	Bedford, Joslyn (1939)	98.5	98.5
Kentucky Wonder	1 yr., -17°	Bedford, Joslyn (1939)	98	100
Blue Lake	4 yr., -21°	Masure, Campbell (1944)	96	—
Beans, Lima				
Fordhook	4 yr., -21°	Masure, Campbell (1944)	96	—
Henderson Bush	4 yr., -21°	Masure, Campbell (1944)	100	—
Brussels sprouts	4 yr., -17°	Joslyn, Bedford (1940)	98.5	85
Cauliflower	4 yr., -17°	Joslyn, Bedford (1940)	97.5	98.5
Corn, cut	4 yr., -21°	Masure, Campbell (1944)	95-98.5	—
Corn, on cob	—	Bullis, Wiegand (1945)	99.5-100	99-100
Peas	1 yr., -17°	Arighi, Joslyn, Marsh (1936)	98.0-99.0	100
Miracle	4 yr., -21°	Masure, Campbell (1944)	50-100	—
Spinach	4 yr., -17°	Arighi, Joslyn, Marsh (1936)	90-100	100

and considerable amounts of ascorbic acid. In recent tests, in which 5 g. of ground vegetable tissue, 5 ml. of water, 1 ml. of 1% tincture

of reagent, and 1 ml. of hydrogen peroxide were used as a base for qualitative test, it was found that the concentration of hydrogen peroxide present initially in the mixture could be varied over a wide range without serious effect on intensity of coloration (see Table XVII). The optimum conditions for enzyme activity when measured either colorimetrically or volumetrically vary with type of color base added as well as with source of enzyme. In using guai-

TABLE XVII

EFFECT OF HYDROGEN PEROXIDE CONCENTRATION ON PEROXIDASE (GUAIALCOL) AND CATALASE ACTIVITY IN FRESH VEGETABLE TISSUE (59)\*

H <sub>2</sub> O <sub>2</sub> , %	Catalase			Peroxidase		
	Asparagus	Peas	Spinach	Asparagus	Peas	Spinach
0.005	±	—	—	—	—	—
0.01	±	++	—	+	—	+++
0.025	+	++	—	++	±	+++
0.05	+	++	—	++	+	4+
0.075	++	+++	±	++	4+	5+
0.1	+++	+++	+	+++	4+	5+
0.15	+++	+++	+	4+	4+	5+
0.20	+++	4+	+	4+	4+	5+
0.25	+++	4+	++	4+	4+	5+
0.30	4+	4+	++	...	4+	5+
0.35	4+	4+	++	+++	4+	5+
0.40	4+	4+	++	+++	5+	5+
0.50	4+	4+	+++	++	4+	5+
1.0	4+	4+	4+	++	4+	5+
2.0	5+	5+	5+	+	4+	5+
3.0	5+	5+	5+	+	4+	5+
4.0	5+	5+	5+	+	4+	5+
5.0	5+	5+	5+	+	+++	4+

\* From data obtained by T. Shearing.

acol for quantitative assay, Masure and Campbell (90) use a mixture containing 0.02% guaiacol, 0.0035% hydrogen peroxide, and vegetable extract equivalent to  $\frac{2}{3}$  g. of tissue per 24 ml. of mixture in citrate buffer of pH 4.5.

For qualitative test under plant conditions we have found the following conditions generally satisfactory: To about 5 g. of cut or crushed vegetable tissue in a test tube, add 5 ml. of water (sufficient to cover) and 1 ml. each of 1% guaiacol and 0.5% hydrogen peroxide

solution. After adding the solutions to the vegetable, the test tube is shaken to mix them and allowed to stand. Note the reaction in two to five minutes.

As in the case of catalase activity, sampling errors, differences in degree to which the tissue peroxidases are made available for reaction, and conditions of the test influence the result. The proper preparation of tissue extracts is an important problem. Qualitatively there are significant differences between enzyme activities in cut tissue or macerates of such tissue and in the filtrates prepared from these—such as those used by Masure and Campbell (90). Although filtration will remove portions of tissues containing thermostable peroxidaselike substances, it will also remove peroxidase activity that may be significant in testing for adequacy of the scalding procedure. Quantitative differences also occur in some vegetable tissues; their significance, at present, is not known. It may be that differences among plant peroxidases exist in the relative degrees to which they are bound to tissue proteins, as is known to occur in dehydrogenases and other enzymes. This is now under investigation.

As shown in Tables III–XII, the pyrogallol peroxidase activity in frozen vegetable tissues, when scalded sufficiently to retain flavor during storage for four years or more at  $-17^{\circ}$ , must be reduced as follows: asparagus, from 0.0055 to less than 0.0002; artichokes, to 0.0000; green beans from 1.13 to less than 0.018; Lima beans, from 0.0073 to less than 0.0002; cauliflower, from 0.0255 to 0.0007; peas, from 0.0080 to 0.000; Brussels sprouts, from 0.0238 to 0.0040; summer squash, from 0.013 to 0.0011; green Italian squash, from 0.0143 to 0.0002; yellow crookneck, from 0.0190 to 0.0015; and for spinach, from 0.0044 to less than 0.0008.

The quantitative methods for estimation of peroxidase activity in frozen vegetables used at present are either based on the determination of intensity of color formed by oxidation of guaiacol (Masure and Campbell modification, 90, of Hussein and Cruess procedure, 53) or on the iodometric determination of the amount of hydrogen peroxide reduced by pyrogallol (modification of Balls and Hale procedure, 8). Neither is entirely satisfactory, *e.g.*, the long period of reaction in the former (90) has been criticized recently (104) and the evidence available at present indicates that the "pyrogallol peroxidase" is not sufficiently specific as a test for scalding efficiency. It is possible that other procedures may be more

sensitive or specific. Iodide oxidation has been proposed as a field test (30) for dehydrated vegetables and may be useful for frozen vegetables. Nadi reagent long used (46) for peroxidase estimation was introduced recently (44,101) in modified form; *p*-aminobenzoic acid (82),  $\alpha$ -naphthol, and other color bases have been suggested.

The most interesting new procedures, however, have been based on the use of ascorbic acid. Since Szent-Györgyi's demonstration (116) that ascorbic acid retarded the rate of color formation of peroxidase reagents it has been demonstrated that peroxidase in the presence of hydrogen peroxide and a suitable phenolic compound will rapidly oxidize ascorbic acid (104), and several methods based on the volumetric or photometric determination of the rate of oxidation of added ascorbic acid have been proposed (38,55,83,104,117). It is possible that they will be more reliable than the methods previously used. Until we have a more specific test for efficiency and adequacy of scalding, based on either the nature of the product or products formed in raw and underscalded vegetables or on the identification of the particular enzyme system or systems involved in off-flavor formation, future developments will be limited to improvements in techniques for peroxidase estimation.

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# INDUSTRIAL BIOSYNTHESSES\*

## Part I. FATS

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### I. Introduction

The expression "biosynthesis" would in Germany be understood to concern the efforts in obtaining protein and fat by means of microorganisms from carbohydrates upon the addition of inorganic nitrogen, mineral salts, etc. These efforts had important meaning during both world wars and in the years before World War II, since Germany is dependent for a portion of her supply of both substances. Regarding these efforts, one might also quote Hildebrandt (30a) concerning the wartime strain on the fermentation industry in the United States: "In industrial fermentation as in other activities the war gave great stimulus to practical research and manufacturing activity."

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\* Translated from the German by S. Hestrin.



From historical as well as purely chemical points of view, it would be desirable to begin with a survey of industrial protein synthesis and then to consider fats. For technical reasons, and in particular due to the present difficulty of obtaining access to the literature, a reverse order of presentation has been selected. Technical advances in the field of protein biosynthesis will be reviewed in a forthcoming volume. In the present review it seems necessary to note that *Torula utilis* Henneberg (*Torulopsis utilis*) has found widespread application in the German protein industry. The status of this subject in 1940 can be judged from an earlier report by the present writer (30).

Progress since that time has been marked, particularly as regards the questions of pentose utilization (see also Sect. VD) and nutritional aspects. It has been found that yeast protein is of lower nutritional value because of its deficiency in cystine. A new method of protein synthesis—the so-called “Biosyn Process” devised by Peukert (58)—is based on the submerged cultivation of molds (*Aspergillus* and others) on waste sulfite liquor of cellulose factories. The increasing significance of industrial protein synthesis even in countries with relatively large resources of raw materials is reflected in the work of Thaysen (84) in England, and in reports (93) concerning an installation in Jamaica that can produce 2000 tons of food yeast per year from molasses (*Torula utilis*). According to Hildebrandt (30a), production of food yeast from sulfite liquor was studied in the United States during World War II by Pederson, Snell, and Frazer. The cost of production of yeast protein was found to be five times as high as that of soybean protein. According to Kurth (30a), greater interest attaches to the possibility of yeast cultivation on factory run-off waters “in order to reduce the oxygen demand of the plant waste and thus lessen stream contamination.”

In the earlier review (30), the author also discussed the history of early efforts to synthesize fats biologically. At that time, the experiments appeared to be insufficiently developed to warrant industrial application. In the interval, the situation has undergone radical change. In the present review, the attempt will be made to place recent advances against the background of earlier investigation upon which they necessarily depend. Considerations of space will limit us to essential aspects and no attempt can be made to present the complete details.

**Note on Nomenclature.** In the German technical literature, distinction is made between *fermentation* (*Vergärung*) and *biosynthesis* (*Zellsubstanzbildung*) under aeration. One refers correspondingly to *fermentation yeast* (*Gärhefe*) and *growth yeast* (*Wuchshefe*). The term *biologische Rohstoff-Synthese* covers both protein and fat, and is used synonymously with "biosynthesis." When yeast is the synthesizing organism, the term *Verhefung* is often employed. In regard to nomenclature of microorganisms, e.g., *Torula* and *Torulopsis*, *Endomyces* and *Endomycopsis*, see Section V.

## II. Historical Note

Production of fat and protein from carbohydrates by the action of microorganisms had already assumed importance in Germany in the first world war. Commercial production of fats by this method first achieved some measure of success, however, only in the second war. In 1942, a leading German industrialist, Krauch (35), was able to make the following statement:

In addition to the purely chemical processes of fat production, we have turned in recent years to biological methods. Microorganisms have been found which exhibit ability to convert sugars and simple saccharides into fat. After considerable initial difficulties, commercial operation of this process has become feasible. Sulfite waste liquor of the cellulose industry is being used as carbohydrate source. The available information suggests that the yeast fat is an excellent foodstuff. With slight alteration of conditions, it becomes possible also to produce yeast protein.

This statement in all probability refers to findings of Schmidt (67), which will be further considered in Section VD. The theory of fat biosynthesis has been discussed by Smedley-Maclean (76) and Bernhauer (3). The present review will be concerned largely with technological aspects. In this connection a brief historical summary is illuminating.

The problem of fat biosynthesis was formulated in the first world war by Max Delbrück, who was then the director of the Institut für Gärungsgewerbe in Berlin and the inaugurator of the method of protein production by the action of *Torulopsis* (*Torula*) *utilis*. Original observations, upon which the process of fat biosynthesis rests, were made by the distinguished biologist Paul Lindner (40, 42) and have been described by him in a publication that appeared

some years after the first war (41). In 1915 Lindner was working with a microorganism that was an unusually vigorous fat former, and he was reminded of an observation made by him twenty years earlier in work on the now generally known method of the hanging-drop culture. "I observed," he relates, "that the plasma of cells in the thin layer of nutrient in the hanging drop becomes progressively more granulated from day to day after the termination of growth, and that the minute individual granules merge into large oil drops when the preparation is allowed to dry out. The yeast culture, in other words, acts as a vigorous fat former." In 1899, Lindner discovered another "fat yeast," a *Torula*, which is named for its characteristic microscopic appearance as "pulcherrima." I can still recall a conversation in 1916 in which Lindner wittily told my father how he had been able to force yeast to form fat until they virtually destroyed themselves by "fatty degeneration of heart." He might have added his later remarks (41): "One might perhaps have thought that the cell would show greater concern for its heritage and desist from overfeeding with fat." The fatty cells are incapable of proliferation. Technical processes of fat synthesis by microorganisms must take this fact into due consideration. In principle Lindner offered a solution of the problem by his choice of a mycelial yeast, *Endomyces* (*Endomycopsis*) *vernalis*. Pilot scale trials with this species seemed promising but a commercial operation of the process was not achieved. Nevertheless, Lindner's observations must be regarded as fundamental to all later successes in this field. (Note that Lindner became aware of the pertinent but very scanty observations of earlier workers—e.g., Nägeli and Löw—only after his own studies.)

The circumstances that led to the choice of *Endomyces* are instructive. Lindner was provided with a dried-out drop of birch sap by one of his former pupils from a field station in Poland. The material yielded a fungus that assimilated but did not ferment sugar and formed large amounts of fat on a sugar medium. The pure culture was identified as *Endomyces vernalis*, first described in 1891 by Ludwig. This fungus appears in the spring on the sap of felled or wounded birch trees as a white mold growth. The fat-forming capacities of this organism were revealed for the first time by Lindner. The plant sap, being poor in carbohydrate, fails to mediate an appreciable accumulation of fat. Thus a basic requirement of fat bio-

synthesis was first recognized: abundant supply of carbohydrate in the growth medium. A second important requirement that Lindner discovered was the need for adequate aeration. (In this connection Lindner suggested an explanation for Liebig's inability to repeat Pasteur's experiments on the cultivation of yeast in solutions of sugars and mineral salts.) He found in addition that the physiological state of the cells requires attention: the inoculum should consist of freshly grown cultures, old fatty cells being unsuitable for this purpose. All three requirements are always encountered in the work of later investigators. Emphasis of this point in the present review has seemed desirable, since it is difficult from our modern vantage point to appreciate the difficulties that beset the early investigations. The case of fat biosynthesis provides an instructive example of a manner by which discoveries can be made, an instance in which necessity forced the association of an accidental event and an overlooked observation to yield a new discovery.

An enormous amount of experimental work remained to be done. The commercial application of Lindner's finding encountered insuperable processing difficulties. Lindner developed two procedures, a "tray process" (*Schalenverfahren*) and a "floor process" (*Tennenverfahren*), both rather unwieldy and requiring an enormous allotment of space. Submerged cultivation, the only method that is technically promising, was unsuccessful. Loss of roughly one-third of the sugar to carbon dioxide represented a further obstacle. (Later study of the chemistry of fat synthesis showed this to be an inherent feature.) The difficulties of surface culture and the prevalence of a sugar shortage in Germany led to the early abandonment of the attempt to operate the process on a commercial scale.

Early reports of the Institut für Gärungsgewerbe were reviewed in 1937 by Fink *et al.* (14), who offer the following comment:

It is the great contribution of Paul Lindner that he was responsible for pioneer investigations of technical value in the field of fat synthesis by microorganisms. Unfortunately, restrictions imposed by the war prevented the publication of the details of this work. The investigation was conducted by Lindner with the help of a staff of investigators including A. Deutschland, R. Erikson, W. Henneberg, K. Nagel, G. Oelbermann, J. Rolle, F. Stockhausen, T. Unger, W. Voltz, and H. Wolf, and is described in extensive archives and protocols. Readers cannot but admire the unsparing devotion and diligence that Lindner brought to bear on this problem. Everything is taken into consideration; every conceivable possibility is weighed in order that a solution

of a great war objective might be achieved. Unfortunately, a practical utilization of this model investigation proved impossible because the costs of the process remained far in excess of the economic limit.

Fink *et al.* (14) were able to confirm Lindner's findings in regard to *Endomyces*, and also extended them to *Oidium (Oospora) lactis*, which seemed to be another promising organism. At that time, *Oidium* was also investigated by Geffers (18,19). In 1937, Fink concluded that commercial operation of fat biosynthesis processes in the near future was unlikely.

In the years preceding and during World War II, numerous unsuccessful attempts were made to establish a commercial process of fat biosynthesis. They resulted in significant contributions to the elucidation of the conditions that this reaction underlies and to our understanding of its mechanism. Among numerous authors the following in particular deserve mention: Bernhauer, Reichel, Rippel, Raaf, Heide, Halden, Nilsson, and Myrbäck,

Despite a long history of failure, the problem was again taken up before and during the second world war by two German industrial groups. They were able to make important forward strides, and independently developed two processes of fat biosynthesis involving submerged culture. Schmidt (67) of the Zellstoff-Fabrik Mannheim-Waldhof used *Torula*, the organism that plays a major role in protein biosynthesis. Applying aeration in submerged culture he was able to obtain yields of 20–30% fat in addition to 25% protein. Damm (6) of Henkel and Company, Düsseldorf, exploited the known ability of fusaria to act as fat formers. An important feature of both new methods and decisive for their technical success was the abandonment of the method of surface culture in favor of a submerged culture method.

### III. Conditions Governing Fat Formation by Microorganisms

Extensive reviews of this aspect have been contributed by Fink *et al.* (14) and Bernhauer (3). Here it is only necessary to consider briefly conditions which govern formation of fat by the commercially promising microorganisms. A more detailed treatment would reach beyond the intended scope of this review.

The basic requisites of fat formation by microorganism were

already recognized by Lindner (41). They are: supply of abundant sugar, excess of oxygen, and use of young, nonfatty cultures as inoculum. *Endomyces vernalis*, the organism investigated by Lindner, assimilates sugars (hexoses except lactose, and pentoses) and does not ferment them. The assimilated sugars are converted above all into cell wall constituents, especially protein and furthermore into fat. All normal substances for the building up of proteins, including ammonium salts and urea, may serve as sources of nitrogen. *Endomyces* initiates growth in culture medium as small islets that link together to a folded pellicle. Fat synthesis sets in after two or three days and is accompanied by the appearance of a characteristic granulation of the contents of the cells. After eight to ten days, fat synthesis is complete: the cells at this time contain a large fat globule, which may be stained with Sudan III. The maximum yield of fat is attained by the fourth or fifth day. While nitrogen is still present in the culture medium, the principal product is protein; this is the phase of the "protein generation." Later the excess sugar, the alcohol, etc., are diverted to fat synthesis, and one obtains a "fat generation." During this phase, the cells lose their ability to multiply. Inocula should therefore consist of young cells of a protein generation taken from a culture medium of low carbohydrate content. Lindner stressed the importance of careful distinction between the protein generation (nitrogen-uptake plus growth) and the fat generation (fat accumulation in absence of any growth). Later investigators (23,61,65,66,79), however, have been unable to confirm the claim that the two successive phases are distinct. According to Raaf (see 61), protein synthesis persists until the complete exhaustion of the carbohydrate component of the culture medium. Nevertheless vigorous protein and reduced fat formation were associated with a high nitrogen medium and, vice versa, vigorous fat synthesis and low protein formation were observed in the low nitrogen culture medium.

In this connection, Steiner (79) and his pupil Raaf (61) introduced an interesting method. The utilization of urea and that of asparagine begins with enzymic liberation of ammonia. If the latter is trapped on permutite, an equilibrium between bound and free ammonia is established and maintained, even though free ammonia is being consumed. Nitrogen remains at a minimum value throughout. One finds, therefore, in otherwise similar conditions about twice as much fat, calculated on a basis of dry mycelium, in a medium containing permutite than in a medium to which quartz sand is added.

In general it was found that fat formation is dependent on limitation of the nitrogen supply, since otherwise protein synthesis tends to predominate (3; cf. also 71). A given ratio of nitrogen to carbon is a requisite. According to Heide (23), the value of this ratio within limits determines the fat content of the cell crop independently of the concentration of the nutrients. Increase of the ratio value is supposed to bring about a lowered fat content, and vice versa. Heide (23) states that a ratio of 7.5% cane sugar to 0.0233% nitrogen is particularly suitable for *Endomyces*. Similar results were obtained by Raaf (61) with *Endomyces* and other microorganisms and by Cada (4a) with *Mucor*. Bernhauer, citing findings of Schmalfuss with flax seeds, suggests that these relations illustrate a biochemical law of general applicability.

It is certain according to Heide (23) that fat is not synthesized from protein. Fink *et al.* (15,67a) earlier supported this conclusion for the case of fat synthesis by *Oidium*.

As for the physiological significance of fat in the fungus as (1) a structural element of living substance, (2) a reserve constituent, or (3) a degeneration or excretion product, Lindner regarded *Endomyces* fat a degeneration product. In *Oidium*, on the other hand, Fink *et al.* (15,67a) consider the fat to be predominantly a reserve substance. According to Heide (23) the same conclusions also hold true for *Endomyces* fat as well. In both fungi, as in animal tissues, protein is spared and is metabolized when the nutrient supply of the medium is exhausted. Raaf (61) has found that fatty mycelium in high nitrogen and carbohydrate-free nutrient solution exhibits a decrease of fat and concurrently a marked rise of crude protein. This suggests that protein can be synthesized at the expense of fat.

Crude protein is the value of the per cent nitrogen multiplied by the factor 6.25. The observation of Dirr and von Soden (77) that an appreciable fraction of yeast nitrogen is in the form of purine derivatives rather than protein is important in this connection. A revision of earlier conclusions concerning the nutrient value of yeast was made necessary by this finding.

Results similar to those of Raaf (61) have been obtained with *Oidium* (see Sect. VD) by Fink *et al.* (15,67a). All these results relate only to surface culture. The data have been exhaustively reviewed by Bernhauer (3). Relatively little information is available concerning the conditions that prevail in submerged fungus cultures.

The same is true also in regard to the effect on fat formation of such factors as *pH* and composition of the growth medium with respect to carbon, nitrogen, salts, and trace elements, as well as the factors of humidity and temperature. According to Schmidt (67), restriction of phosphate in *Torula* leads to a reduction in protein and to a rise in fat. The following comment from a patent of Henkel and Company seems to be apt in all these questions (26): "A culture formula that would be universally applicable does not exist."

Considerations relating to the theoretically possible yield limits have been presented by Rippel (65). He designates the sum of the products obtainable from 100 g. of carbon nutrients as the "economic coefficient," and on the basis of energy calculations finds that the "fat coefficient" (fat formed from 100 g. of sugar present) cannot exceed, roughly, 15. Detailed analyses of these questions and numerous accompanying tables have been presented by Bernhauer (3). He reaches the following conclusion:

Theoretical yield limits of industrial fat biosynthesis by microorganisms would thus appear to be fixed. For mycelium with a fat content of 30%, the maximum possible yields from 100 parts of carbohydrate are 15 for fat, 10 for protein, and 20 for other cell constituents. For mycelium with a fat content of 50%, the fat yield rate being maintained at the same level, *i.e.*, 15, the maximum possible yield of protein is only 3 and of other cell constituents 10.

According to Damm, this conclusion, which was derived in the first instance for surface culture, is equally true for submerged cultures of fusaria. In this case, too, the theoretical maximum yield of fat is said to be 15.

#### IV. Survey of Industrial Cultivation Methods

Industrial fat synthesis by action of microorganisms is governed by the manner by which the following requirements are met (*cf.* 71): (1) Choice of a suitable microorganism to ensure maintenance of a high and consistent yield. (2) Establishment of a cheap but suitable culture medium. Substances high in pectin and cellulose, *e.g.*, wood wastes and sulfite liquor, are indicated in this connection in preference to the more conventional substrates (starch, sugar, and molasses). As in the case of protein synthesis, pentose utilization is a factor to take into due account. (3) Development of suitable cultivation. The commanding importance of this aspect is evident from our brief review of the history of the industry (Sect. II).



These requirements are met in all similar cases. Especially, the choice of a suitable organism is of great importance. In Section V, the methods of fat biosynthesis will be reviewed according to the species of microorganism. This classification provides a convenient framework also for the discussion of substrates. It seems useful to precede the detailed description by a brief general survey of cultivation techniques. In some cases, *e.g.*, *Torula*, the choice of a suitable method was decisive for technical success.

Since fat synthesis is an aerobic process, three general methods of cultivation may be taken into account: (a) Growth on surfaces of liquids—"Tray Method." (b) Growth on solid surfaces, or on surfaces of carriers moistened with nutrient solution. This category comprises cultivation on floors of malt houses or similar areas—"Floor Method." (c) Submerged growth in aerated tanks. It is evident that the method of submerged cultivation, having a lower space requirement, reduced contamination hazard, and smaller labor requirement, is a technique of choice. The costs of aeration, on the other hand, are higher as in the case of the tray method, but the costs of equipment are lower. Many investigators have accordingly concentrated on the submerged culture. But most of them did not succeed. To a certain extent, their failure was influenced by the inability of their microorganism either to grow well in submerged culture or to produce an adequate amount of fat in these conditions. In part they did not govern the equipment difficulties. Only two processes have led to technical success: The previously cited Schmidt method, which was used by Mannheim-Waldhof to grow *Torula*, owes its success to a special aeration procedure, "Mannheim's emulsion process." In Damm's process, relatively simple types of equipment are used. The effectiveness of his method stems from the recognition of the fact that fusaria are equally capable of fat synthesis in submerged as well as in surface culture.

#### A. GROWTH ON SURFACES OF NUTRIENT SOLUTIONS

The tray method (14,40,41) was developed in 1916 and 1917 in pilot trials by the Institut für Gärungsgewerbe of Berlin, Behringwerke of Marburg, and Melassefabrik C. Meyer of Danzig. For a while, the experimental installation at Berlin turned out 10 kg. of fresh fungus mycelium (*Endomyces*) per day. The nutrient medium in a layer of 1-2 cm. was placed in enamel or magnesium-aluminum

alloy (magnalium) trays each with an area of 0.17 m.<sup>2</sup> The trays were in stands arranged to permit unhindered air circulation. The successful operation of this technique required maintenance of a proper ratio of surface to volume. After two to three days at 20°C., the nutrient solution under the formed pellicles was drawn off and replaced by fresh solution. Caution must be exercised in this operation to avoid wetting the pellicle, since wetting would hinder proper access of air. The maximum growth was attained after five to seven days. The undersurface of the pellicles was washed by repeated rinsing. The mycelium was converted into a paste, or dried and extracted after suitable treatment. The difficulties of the technique are all obvious: high cost of equipment, high labor demand, and high contamination liability. Similar problems were later investigated in reference to *Oidium lactis* by Fink *et al.* (15,67a) and to *Penicillium javanicum* by Ward *et al.* (87) in America. Since *Oidium* is a relatively resistant group of organisms, its use circumvents the serious hazard of contamination, which forced the abandonment of *Endomyces* production by Behringwerke in 1917.

Among the various attempts that have been made to improve the tray process, the patent of Klapproth (34) is noteworthy. The latter describes the use of floating membranes, to serve in the place of fine sieves for lifting the fungus pellicle. The membrane may consist in part of substances that contribute to the growth of the fungus.

## B. CULTIVATION ON MOISTENED CARRIERS

Linder's floor method of World War I was devised partly with the idea that it would provide a use for the then empty malt houses. The *Endomyces* was cultivated on carriers of an indifferent nature, *e.g.*, chopped straw, reeds, rushes, coarse wood chips, potatoes (Ger. Pat. 310,616), and the like (40). This material is freed from dust, rinsed, moistened with nutrient solution and then sterilized and inoculated with a nutrient solution containing the fungus in even distribution. The loosely packed heaps were turned over at frequent intervals; at these times they were also moistened. Growth was complete at 11°C. after about twelve days; at 20°C. it was complete within eight to ten days. The product was dried at 50°C. in malt kilns. The method, although simpler than the tray method, does not permit equally complete utilization of sugar. Separation of the fungal

crop from the medium and the isolation of the formed fat are also apparently more difficult.

In the course of this work, the applicability of Topf malt germinators to *Endomyces* cultivation was examined with negative results. Among other difficulties, infections caused by *Torula*, proved troublesome.

Mention may be made of later patents of Stöb in which elaborate devices involving surfaces with a slight forward incline are described. Bernhauer (3) does not believe that these proposals are feasible on an industrial scale.

Methods involving the exposure of yeast on large surfaces to alcohol vapor have received attention. Successful results can only be obtained in the process if extremely thin yeast layers are used. Otherwise, differences of fat content ranging from 30–40% (dry weight basis) in the upper part to 15% in the bottom part are encountered. A process of this description has been patented recently by Halden (22). His method has serious technical difficulties but is interesting in its theoretical implications. The data are consistent with the belief that acetaldehyde plays a central role in fat synthesis. Lindner (41,43) was the first to draw attention to this possibility. In a patent (Ger. Pat. 332,411), he proposed (40) the use of vertically hung cloths soaked with inoculated nutrient solution and exposed to vapors of alcohols or alcohol derivatives in conditions of adequate air supply. Acetaldehyde was used by Lindner (43) and later by Ehrlich (9). The observation of Halden that fat formation in yeast is favored by lowering of water content in the presence of alcohol is particularly interesting in the light of the increase of fat in oil seeds of higher plants and in the bark and wood of oil trees after exposure to drought.

### C. SUBMERGED CULTURE

Bernhauer distinguishes four methods of submerged cultivation: aeration, shaking, stirring, and the revolving drum method. The technique of shaking is not economically workable. The revolving drum method has been used industrially for other purposes, *e.g.*, the biosynthesis of citric acid (30). Its suitability to fat synthesis is undetermined. The writer does not consider the strict differentiation of aeration and stirring techniques to be a useful one.

Mechanical devices for aeration and stirring in fermentation tanks

have been the subject of a number of patents. Their applicability to fat synthesis is undetermined. Considerable discussion has been devoted to the relative value of different aeration types, *e.g.*, Stich's method of fine aeration, and the Vogelbusch method in which use is made of a tubular winged stirrer with numerous tiny orifices.

In Lindner's experiments on yeast, considerable yields of fat were obtained when an excess of sugar was allowed. Utilization of this procedure on an industrial scale was unsuccessful because of strong foaming.

The successful Schmidt process (67) of Mannheim-Waldhof involves a special aeration technique. In this case, a virtue is made of necessity: the difficulties of foam control that forced earlier investigators to abandon submerged cultivation are circumvented by a process in which the solution can and must foam. The nutrient liquid is converted into a very fine emulsion with air by a procedure also used at Mannheim-Waldhof for the production of *Torula* protein from sulfite liquor. The aim in that case was to devise a method that would not require an addition of fat as an antifoaming agent. The latter a by-product of wool production, was difficult to obtain in Germany during the war, and moreover, had the further disadvantage that its addition to the growth medium gave the yeast a characteristic rancid taste and odor. According to Schmidt (67), two methods that have enjoyed a similar degree of success have been found: the Waldhof method and the I. G.-Scholler method. For some time, the two have been shared under mutual agreement between the parties concerned and have been collectively known as the "Waldhof-I. G. Process." Schmidt (67) describes them as follows:

In the Waldhof process, the waste sulfite liquor of beech wood pulp is allowed to foam and is gradually converted into a fine air-liquid emulsion. A particularly favorable oxygen supply is thus ensured, as is shown by the fact that an expenditure of 8-10 m.<sup>3</sup> of air per kilogram of yeast was adequate, whereas 25-50 m.<sup>3</sup> had been required in the continuous flow process of 1940. Satisfactory distribution of the yeast is effected by means of a continuous rolling around. Using a continuous process, the time requirement for yeast production is very low.

The I. G. Farben process also used the experiences of Scholler with yeast production from wood sugar, and has been developed both for waste sulfite liquor from beech wood and for distillery wastes. The method employs a novel aeration principle, introduced by Seidel, a co-worker of Scholler, which delivers an easily liquefying foam of high liquid content. The aeration units

consist of a series of toothed rings through which finely divided streams of air are expelled. The sulfite liquor is brought to vertical circulation by air guided by means of air pipes, which are supplied at their lower ends with a Seidel aeration unit. The liquid at the bottom of the tank is displaced upward according to the principle of Mammut pump and is thrown in a powerful stream on the foam at the surface. By this means, the foam head is broken and addition of fat as an antifoaming agent may be dispensed with. For further details, see Section VD.

According to a patent of Mannheim-Waldhof (see 92), 7 m.<sup>3</sup> of liquid yields 30 m.<sup>3</sup> of foam. The foam weight is therefore 250 g. per liter. Since the microscopic air bubbles are practically static with respect to the liquid, the microorganisms have enough time to make full use of the available oxygen supply. A foam head does not form. (Cf. also in this connection the patent of Scholler and Eichemeyer, 70, and that of Seidel, 75.)

A patent of Stöb (80) describes a further foaming process. Numerous other patents have been issued to Stöb (81), the merits of which the present writer cannot judge. The same applies to the process of Müller (47) in which conversion of the culture medium into a foggy emulsion (*Nebelform*) is described.

Although submerged cultivation of yeast has been practiced successfully since olden times, cultivation of mycelial fungi by the same technique has often proved unsatisfactory. Low yield, clogging of aeration orifices by the fungus, and excessive foam formation in the case of certain types of substrate, *e.g.*, whey, have been some of the difficulties described. The species of microorganism is important in this regard. Fungi such as *Endomyces vernalis* have a definite preference for undisturbed growth conditions and perform badly in tank trials for this reason. *Oidium* yields are 3 to 4 times higher in an undisturbed than in a submerged culture. The success of the Damm process undoubtedly owes a great deal to the choice of a suitable microorganism. "Among different microorganisms that seemed suitable to submerged culture as a matter of principle, *Fusarium* was selected for technical investigation because it possesses a number of advantages" (6). The nature of these advantages has not been mentioned. In its final form, the Damm process involves use of a closed tank system with fine and coarse aeration and seems to have a stirring device. Difficulties that the submerged cultivation of mycelial fungi had presented to earlier workers seem not to arise at all in this case.

## V. Fat-Forming Microorganisms

In the following paragraphs, processes described above in general terms will be discussed with reference to specific microorganisms. No attempt will be made to follow any formal bacteriological system of classification (*cf.* Niethammer, 49a). An organism will be designated in general by the common name used in the older technical publication. The modern scientific nomenclature of yeasts, as inaugurated by Stelling-Dekker as well as Diddens and Lodder (79a), is indicated in parentheses. This nomenclature is used today in Germany (*cf.* 49a,66a) and is already discussed in the United States (*cf.*, for example, 59a).

Harder and Witsch (22a) have found that certain diatoms synthesize considerable amounts of fat in the absence of added organic nutrients. Since only an abstract of this paper has been available, it will not be discussed here in further detail.

### A. *ENDOMYCES VERNALIS* (*ENDOMYCOPSIS VERNALIS*) LUDWIGII

*Endomyces vernalis* is an ascosporeogenous yeast and forms ascospores with or without isogamous copulation. In beer wort its growth



Fig. 1. *Endomyces vernalis* (21b).



Fig. 2. *E. vernalis* with fat droplets (21b).

leads to the formation of a folded pellicle. The septated mycelium contains cells of  $2-5 \times 20-60 \mu$  and oidia of  $3-4 \times 8-15 \mu$ . After inoculation daughter cells form rapidly and grow as a filamentous mycelium. According to Günther and Bonhoeffer (20; see also Sect. VII), filaments of the protein generation may be  $2 \mu$  thick and up to  $30 \mu$  long, with individual cells up to  $5-10 \mu$  long. In the fat genera-

tion the cells of the filaments round out to a diameter of 5–10  $\mu$ ; the cell contents granulate and large oil drops appear. Excellent illustrations of the process of fat accumulation in *Endomyces* have been presented by Haehn (21b); see Figures 1 and 2 from photographs by Lindner.

Experiments relating to the commercial use of *Endomyces* have been considered in Section II. The work of Lindner during World War I was described only briefly in the patent literature (40), and in short papers (41,42), both published after the end of the war. The experimental details were made public much later by Fink and Haehn (14). The claim of the basic patent (Ger. Pat. 306,365) granted to Lindner (40) is the most important of the numerous patents assigned to this investigator:

Process for production of fat by cultivation of mold fungi on solutions of carbohydrates and mineral nutrients (*e.g.*, molasses, beer wort, and similar plant extracts) which consists in multiplication of the fungi either on liquid media of large surface or on carriers moistened with nutrient solutions and possessing a large surface.

This claim contains the description of the tray as well as the floor method. In connection with the differentiation of two generations (see Sect. II), the following is mentioned:

At the termination of the vegetative period, the fungi are given a starvation treatment in the presence of an abundant air supply and reduced humidity.

Use is made particularly of aerophilic molds and yeasts, *e.g.*, *Oidium sachsia* and *Endomyces* strains. The cultivation of *Endomyces vernalis* on sulfite liquors was described two years later in patent 305,091 (40). This was the first approach to an economically feasible method. Besides sulfite liquors to which beet juices could be added, whey and similar sugar-containing, nitrogen-low wastes are mentioned as possible substrates. Fink *et al.* (14) report that the protocols included mention of molasses and cellulose waste liquors under this category.

On molasses alone, the fat coefficient (g. fat/100 g. sugar) for the tray method was 10–12, the mycelial fat content being up to 45%. In the floor method, the fat coefficient was 7.6. Sulfite liquor alone yielded 4.8 g. fat from 100 g. sugar. With autolyzed yeast as protein supplement, the yield from 8.91 m.<sup>3</sup> of sulfite liquor on a tray area of 758 m.<sup>2</sup> was 100 kg. dry mycelium or 33.88 kg. fat. No data are given as to yields obtained with the other substrates. In view of the

sparsity of sugar in the liquor, it is necessary to assume according to Lindner that fat is also formed from amino acids. According to Fink, this is in agreement with the findings of other investigators. The calculation of yield on the basis of sugar is therefore attended by a measure of uncertainty.

Cultivation on sulfite liquors is relatively free from the infection dangers that beset cultivation on molasses, but the presence of specific protecting agents can be suspected.

According to Reichel and Schmid (62,63), *Endomyces* in pure nutrient solutions converts 21.5% of glucose and 24.8% of fructose into fat.

Fink *et al.* (14) describe a procedure used by Lindner to produce 50 kg. dry fungus per day. The following were required: 5.2 m.<sup>3</sup> sulfite liquor, 3.8 kg. technical grade urea, 1.9 kg. superphosphate, 2.5 kg. potassium chloride, and 1.25 kg. magnesium sulfate. The sulfite liquor is diluted to 10° Balling. In 72 hours, at 18–20°, the protein generation is produced in the form of a continuous crinkled pellicle. The nutrient fluid is then drawn off and replaced by neutralized sulfite liquor diluted to 18° Balling. Two days later, fat formation has terminated. The process clearly meets the essential requirements: a protein generation on high nitrogen and relatively low carbohydrate medium; a fat generation on high carbohydrate medium in the presence of an abundant air supply (extensive surface) and an optimal temperature of 15–20°C. The fat cannot be extracted directly. It is necessary either to subject the cell material to a preliminary "unlocking" treatment with dilute warm hydrochloric acid, or to allow autolysis to proceed for two to three days at 50°C. as described by Lindner in patent 309,266 (40). The protein in the defatted residues can be extracted by boiling in water and used as a nitrogen supplement in the culture medium.

As has already been mentioned, the process enjoyed but questionable technical success, and appeared to be of interest only as an emergency war measure. High initial costs and the high cost of the labor involved in the tray method made it uneconomical. After the termination of the war, the process was abandoned. A valuable contribution in the field of the chemistry of fat synthesis was made at this time by Haehn and Kintoff (21) (see Sect. VII). *Endomyces* was also subsequently studied again by a number of other investigators.



Nadson and Konokotina (48) have described a series of experiments on fat production by *Endomyces vernalis* conducted as a state project in Russia during World War I. The results were very similar to those obtained earlier by Lindner. It could be shown that *Endomyces* prefers an undisturbed medium; an aeration of the nutrient solution (beer wort) led to unfavorable results. In opposition to Lindner's findings, the Russian investigators reported that lactose too could be assimilated. Their observation that small amounts of lactic acid exert a favorable effect is consistent with Heide's later work (23), in which it was found that *Endomyces* growth is optimal at pH 4.5 (limiting range of pH, 4-6). The temperature optimum was 15°. At 27° growth was already depressed, and at 37° it was completely inhibited.

A kind of floor method was likewise developed. A culture of *Endomyces* was inoculated on to diced potatoes that had been sterilized at 120°C. fifteen minutes. After eight to fourteen days, the yeast had a high fat content. In the best case, using frozen and rotted potatoes, the product contained 9% fat after a growth period of seventeen days.

#### B. *OIDIUM LACTIS* (*OOSPORA LACTIS*)

*Oidium lactis*, the so-called milk mold, forms a thick opaque yellow pellicle on sour milk. Its filamentous mycelium lacks special fruiting bodies. The filaments may be divided into chains of short rounded-off cells which can break free. (This is the typical formation of so-called "oidia" or, better, "arthrospores.") *Oidium* is responsible for the early stages of reddening in certain soft cheeses (30). Utilization of its marked capacity to accumulate fat in surface culture was first proposed by Lindner (40) in World War I (Ger. Pat. 306,365). Technically results with *Oidium* were no more encouraging than with *Endomyces vernalis*. Growth and fat synthesis in whey culture were studied in 1936 by Geffers (18,19) at the suggestion of Damm (6) and by Fink, Haeseler, and Schmidt (15,67a). The technical results were again negative.

In a total of fifty investigated strains, Fink *et al.* found ten that possessed particularly marked ability to synthesize fat. Of these, two were also distinguished by a high proliferation capacity. Designated as A and B, they are grown respectively, in 4 and 6% sugar solution in nutrient media, chiefly whey, at 25 to 30°C.

Since *Oidium* mediates complete assimilation of lactic acid, the medium becomes alkaline. The relative yields are higher than are obtained with *Endomyces*. Fink does not consider the formed fat to be a degeneration product in the sense suggested by Lindner and Henneberg, and regards it rather as a reserve substance. Geffers (19), on the other hand, supposes it to be a degeneration product since it is incapable of metabolic conversion.

Important strain differences were also found by Geffers. This investigator differentiates two groups of fat formers: one group that utilizes lactose, glucose, and galactose, but not lactate, and forms a mycelium that contains up to 50% fat (dry weight basis), and a second group that does not utilize lactose, assimilates lactate as well as glucose and galactose, and forms a mycelium containing only up to 15% fat (dry weight basis). *Oidium* is less susceptible to infection than *Endomyces*. The milk mold is capable of growth on unsterilized whey, which already contains lactic acid bacteria. Despite this advantage, serious difficulties were encountered. The objective of submerged cultivation in tanks could not be achieved. The use of the tray method again resulted in an excessive space requirement. According to Fink's calculations, 10,000 m.<sup>2</sup> tray surface, with 70 crops per year, should suffice to work up 10,000 m.<sup>3</sup> of whey and yield 200 tons of dry fungus or 53 tons of fat. The defatted residues, amounting to 147 tons and containing 23% crude protein, serve as stock feed. The high initial outlay cost makes the process of doubtful economic value.

Fink *et al.* (15,67a) found 22.5% fat, on a dry weight basis, in crops harvested after six days. Within this time, the consumption of lactic acid sufficed to shift the pH reaction from 4.2 to neutrality. After longer cultivation, the fat content fell. The fungus was grown in Jena flasks containing 250 ml of a culture medium of the following composition: 2 liters of whey, 2 g. of ammonium sulfate, 1 g. potassium biphosphate, and 0.5 g. magnesium sulfate. Growth and dry weight continued to rise until the seventh day. The fat content increased during this time from 7.5 to 22.5%. Concurrently the crude protein content decreased from 33.75 to 7.56%. The pH rose in the course of six days to the neutral point. With further prolongation of culture, the medium became decidedly alkaline (up to pH 8.3), the fat content decreased, and the protein content increased (cf. 61; see Sect. II). The nitrogen level of the medium

continued to decrease up to the sixteenth day, and it can be assumed therefore that there was no appreciable decomposition of mycelial protein.

Geffers worked with *Oospora* Wallroth growing on whey optimally at a pH of 5.4 to 5.9. Another group showed its optimum growth at pH 5.1 to 5.4. Submerged growth with aeration produced unsatisfactory results. The method of shaking was equally poor. With surface growth, 50 ml. of whey afforded 104 mg. dry substance containing 14.4% fat in four days, and 637 mg. dry substance containing 157 mg. fat in 120 days, i.e., a yield of 24.6%. With another strain, 45.3% of fat was found in the dry substance after 55 days. Periods of such long duration are of course uneconomical.

*Oidium lactis* can be cultivated on sulfite stillage (31) as well as on beech wood waste liquors (32). In both cases, using the tray method the pentoses are also utilized.

In the case of beech liquor, 100 liters containing 1.9% sugar and a supplement of 0.05% diammonium phosphate and 0.1% urea yielded 1.3 kg. dry mycelium in two days. The fat content is not given in the patent description.

Fink *et al.* (15,67a) showed that *Oidium* is capable of pentose assimilation as are fusaria. In an industrial process Seidel (74) put this property to use in manufacturing an organic nitrogen nutrient to be employed as an admixture to fermentation media for the production of microorganisms or fermentation products: in a medium containing hexoses and pentoses, the hexoses are first fermented in the standard way; second, a microorganism that assimilates pentoses is grown in the solution; third, these organisms are then plasmolyzed. The pentose-assimilating organism is *Oidium lactis*. The plasmolyzate may furthermore be employed with profit in biological protein synthesis by *Torula*.

Fredholm (cited by Waeser and Schenk, 86) found maximal yields with *Oidium lactis* on whey on the fifth to the sixth day of cultivation; longer cultivation led to a decrease in the fat content. The fat yield could be increased by cultivation in symbiosis with different lactic acid bacteria. The latter were introduced about forty hours after inoculation with *Oidium*. With supplement of *Streptococcus lactis* or *cremoris*, 42% fat on the basis of dry substance was obtained.

Recently, Popowa and Putschkova (58a) investigated the formation of fat by a special culture of *Oidium lactis* in whey (1–1.5 cm. layer). The mycelial weight is increased by sodium chloride, ammonium sulfate, ammonium diphosphate, asparagine, and urea.

The formation of fat is also increased by the same substances, except for ammonium sulfate. After 18 to 20 hours at 22–27°C. the mycelium contains up to 20–23% fat. If the lipase is inhibited by oxidative means, *e.g.*, hydroperoxide or potassium bromate, the formation of fat is increased. (See also Section VII.) Addition of proteinase-like enzymes also increases fat formation as well as dry matter yield. The characteristics of the fat are: acid number, 145–170; saponification number, 180–210; iodine number, 35–55.

*Oidium* fat is bright yellow to brown in color. Strain A of Fink yields a petroleum-jelly like product; strain B yields an oil. As in the case of other microorganisms, the properties of the fat vary, depending on the conditions employed (see Sect. VI).

### C. *PENICILLIUM*, *ASPERGILLUS*, *MUCOR*

A number of theoretical studies have been devoted to the factors governing fat formation by these fungi. A detailed review has been presented by Bernhauer (3). It is noteworthy that Pruess, Eichinger, and Peterson (60), who surveyed numerous strains of *Aspergillus* and *Penicillium* noted variations in fat content ranging from 1.1 to 24.4%. Large fluctuations are also observed within any given strain. The fat content of different strains of *Aspergillus fischeri*, for example, fluctuated between 10 and 20%.

In experiments that appear to have been carried out with the possibility of eventual industrial application in mind, Ward, Lockwood, May, and Herrick (87), using a shallow pan fermentation technique and an aerated incubator of special design that supplied 4–5 liters of air per minute, have shown that the fat content of mycelium of *P. javanicum* can be as high as 41%. As a by-product of this process, citric acid is formed in considerable amount. The sugar utilization is low; the fat coefficient is only 5–6. The type of the fat obtained depends on the composition of the growth medium. The product from a medium containing 30–40% glucose has an acid number of 50–55; that from medium containing 20% glucose has a value of 10–15, *i.e.*, the free fatty acid content is in this case lower (*cf.* Section VI).

Maximum yields were found when 12 liters of medium containing 30% glucose, 0.225% ammonium nitrate, 0.03% potassium biphosphate, and 0.025% magnesium sulfate were used per tray. After nineteen days of growth, the yield of mycelium amounted to 445

g. dry matter containing 106.5 g. (23.9%) fat and 249 g. citric acid. The consumption of glucose amounts to 1850 g. The economic coefficient is thus 24 and the fat coefficient 5.75 only. The yield as in any tray process varies according to the surface/volume ratio. If the layer thickness of fluid medium is reduced from 9.6 to 2.4 cm., the surface/volume ratio being increased thereby from 0.103 to 0.413, the yield of fat per gram glucose is increased from 32 to 52 mg. and the fat content of the mycelium from 16 to 21%.

Employment of *Mucor* species was proposed by Portheim (59). whose Austrian patent claims rights for the cultivation of *M. spinosus* and *stolonifer* on sugar solutions, especially wood sugar, at a concentration of 3%. In connection with this process (concerning its technical efficiency, nothing is known) Blinc and Bojec (4) studied *Mucor mucedo*. In laboratory trials of growth on sulfite liquor, they obtained a fat with an abnormal iodine number of 125.6 in 6.5% yield, on the basis of mycelium weight. This represents a fat coefficient of 1.3 and an economic coefficient of 20. According to unpublished experiments by Bernhauer (3), *Mucor* species grown in submerged culture may contain more than 50% fat, the fat coefficient being 12–15 and thus approaching the theoretical maximum of 15 calculated by Rippel (65). Patents assigned to Henkel and Company (26,27) refer to experiments on submerged cultivation of a variety of molds, including *Mucor* (see Sect. VE). Mixed cultivation of *Fusarium* and *Mucor* species has been discussed by Niethammer (see Sect. VE).

#### D. SACCHAROMYCES, TORULA UTILIS (TORULOPSIS UTILIS), RHODOTORULA GLUTINIS

The ability of domestic yeasts to form fat was studied in Berlin during World War I in conjunction with the work on *Endomyces*. With small inocula and large excess of sugar, fat formation was found to be small. With large inocula, however, fermentation was so intense that large scale trials did not appear to be justified.

In an extension of earlier work by Lindner and Unger (43). the ability of yeast to convert alcohol to fat was investigated. Even the ordinarily poor fat formers e.g., bottom brewery yeasts, press yeasts, and *Torula utilis*, rapidly form fat from alcohol. Fink, Haehn, and Hoerburger (14) cite an experiment by Lindner in illustration of the method in which a paste of bottom yeast was spread in a thin

layer on the walls of a 100 ml. flask containing a 4% aqueous solution of alcohol at its bottom. Judging on the basis of microscopic appearance, the cells contained 30 to 40% fat at the end of a period of 48 hours. Ethyl alcohol can be replaced by either methanol or paraldehyde. Experiments with clay and glass surfaces on a pilot scale have been reported by Fink *et al.* (14). Technical possibilities of this method were also investigated by Halden (22), who has made the novel observation that the sterol content of *Saccharomyces cerevisiae* may be increased sixfold if the water content of the cell is reduced.

Results of economic significance in the use of the yeast *Torula utilis* for fat production were first obtained during World War II by Schmidt (67) of Mannheim-Waldhof. The process developed involves the emulsification of the growth medium with air (see Sect. IVC) as in the case of the synthesis of protein by yeast. The conditions that are employed are said to favor the parallel formation of both fat and protein, rather than a secondary formation of fat from protein. The yeast is maintained throughout its growth in the same optimal medium. It is noteworthy that Schmidt discusses the process in terms of protein-fat conversion, although this concept had once been rejected in favor of the assumption that the fat is derived exclusively from carbohydrate. The method is described by Schmidt (67) as follows:

The crude liquor from the cooking of a wood pulp is brought to the desired acidity by addition of lime or calcium carbonate, the amount required being slightly different for beech liquor than for pine liquor. Nutrient salts (nitrogenous substances, potassium, magnesium, and phosphate) are then added and the cooled liquor is fed into the yeast vats at a steady rate. Heat evolved as a result of vigorous vegetative processes is drawn off by means of cooling coils. At Waldhof, old fermentation vats 8 m. in diameter and 6 m. in height are used. The revolving aerator is at the bottom of the vat beneath a stationary cylinder. It draws air from outside through a central pipe and emulsifies it with the liquor. The weight of the emulsion depends on the surface tension. Beech liquor emulsion, for example, weighs about 250 g. per liter (*i.e.*, 1 liter of emulsion consists of 250 cc. liquid and 750 cc. air), pine liquor emulsion about 400 g., and wood sugar emulsion about 500 g. The emulsion formed by the aerator at the bottom of the vats is propelled outward, rises high along the walls, and falls back gently onto the central cylinder. A continuous circuit is thus established. The evaporative loss of air to the atmosphere is replaced by new emulsification. Yeast production is continuous over a period of weeks in the course of which fresh liquor is fed

into the vat through the central cylinder and yeast and stillage are removed in equivalent amount. The rate of flow is adjusted to allow a contact time of less than five hours for beech liquor, and roughly three hours for pine liquor. The temperature is maintained at 32–34°C. After deaeration in a specially constructed centrifuge, the material is passed through a rapid separator. The resulting product containing 10–15% yeast is diluted with water and

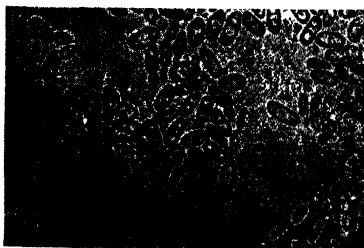


Fig. 3. *Torula*,  $7 \times 3 \mu$ , with large vacuoles and protein between the cell walls and vacuoles (ca.  $\times 640$ ) (67).

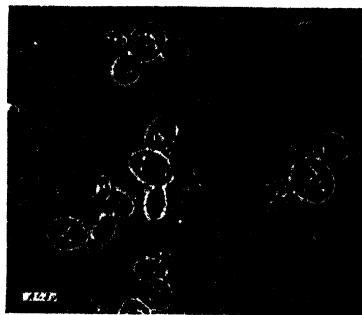


Fig. 4. Fat yeast with fat droplets that are partly bright and partly dark (ca.  $\times 1000$ ) (67).

centrifuged to free it from remaining liquor. After removal of water by filtration, the yeast paste is liquefied by thermolysis and pumped into an evaporator where it is concentrated to 30% dry matter. Drum or spray driers are employed to produce dry yeast with a water content of 5–8%.

The above description refers evidently to protein production and is subject to modification for fat. Varying the nutrient (p. 678), the contact time is increased to ten hours. Details concerning the isolation of the fat have not been offered. Extraction of the fat from the intact cells, according to several other investigators, presents a number of serious difficulties (see Sect. VI).

The process of the synthesis of fat rests on similar biological principles to those underlying the production of protein by the action of *Torula*. The basic investigations, contributed by Fink (10,11), will be considered in detail in discussions dealing with protein production to appear in a forthcoming volume (see also, 30). Some necessary remarks will, however, be given here.

*Torula utilis* is an anascosporogenous yeast with oval hyaline cells. Since sexual fruiting bodies have not been found, the organism is regarded as an imperfect fungus. The cells are generally separate,

and rarely yield mycelia. On beer wort agar, the colonies are a glossy grayish white. A large variety of sugars is fermented.

*Torula*, in contrast to domestic yeasts, shows a marked Pasteur effect (compare 2 and 44). Its metabolism can be switched almost completely from fermentation to respiration and synthesis when it is cultivated on a suitable medium with abundant aeration. Then "alcoholic fermentation becomes an insignificant side reaction, and cell proliferation as biological synthesis strongly predominates" (see 10). The use of sulfite liquor is based on the findings of Fink and Lechner (11,38) that *Torula utilis*, like *Fusarium* (51; see also Sect. VE), is able to utilize pentoses, a conclusion indicated also by the earlier demonstration that the yield on waste liquor is greater than can be accounted for on the basis of hexose utilization alone. Lechner (38) summarizes his experience in the following terms:

Early attempts to grow *Torula utilis* on xylose failed, that is, increase of the yeast was not secured despite disappearance of the xylose in the course of aeration. In October of 1938, however, the surprising result was obtained that *Torula utilis* can be grown on aerated xylose medium.... Production of yeast from xylose was not subsequently attended by any difficulty. The reason for the changed result was never discovered.

See also Windisch, 88, referred to at the close of this section (see p. 679).

Later it was found possible to grow the cells on beechwood liquor. This finding was of special significance because of the enormous increase in the use of beech by the German cellulose industry after 1937. Sugar produced from leaf woods (130,000 tons\* per year) was unsuitable for alcohol manufacture, and its disposal formed a problem of some urgency. The reducing substance of wood sugar of pinewood contains 80% hexose and 20% pentose; that from leaf woods contains 60% hexose and 40% pentose. The reducing material of sulfite liquor of pinewood contains 75% hexose and 25% pentose; that of sulfite liquor of beechwood contains only 10–15% hexose but 85 to 90% pentose. Distiller's wastes contain pentoses exclusively. The importance of utilization of pentose by *Torula utilis* is self-evident from a consideration of these figures.

A second important finding was the observation that *Torula utilis* utilizes acetic acid, which is present in larger amounts in beechwood liquor than in pinewood liquor (35–40% on the basis of the reducing substance in beechwood liquor as against 15% in pinewood liquor).

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\* 1000 kg./ton.



This accounts for notably improved yields of yeast from sulfite liquor and distiller's wastes.

As has been pointed out, the above findings were made originally in connection with protein biosynthesis. They are equally relevant, however, for fat synthesis. The following comment is made on the basis of the data of Schmidt regarding energy requirements. Synthesis proceeds in the presence of oxidation reactions. Combustion of sugar must provide the energy required for the endothermic syntheses. The amount of heat produced in respiration is almost forty times that produced in fermentation. At least one-third of the carbon is lost as carbon dioxide. The considerable amounts of heat evolved during vigorous growth must be drawn off by efficient cooling systems since otherwise the course of the reaction is completely altered. Assuming that one-third of the total carbon is converted into carbon dioxide, the maximum yield per 100 g. glucose containing 40 g. carbon is 26.7 g. carbon as yeast and 13.3 g. carbon as carbon dioxide. Since the carbon content of yeast is 45%, the theoretical yield would be 59 g. yeast and 49 g. carbon dioxide. In carefully conducted laboratory trials, Fink obtained yields that equaled 89.2% of theory. This result is of the same order as that found in alcoholic fermentation, which likewise diverges from the theoretical formulation of Gay-Lussac due to interfering side reactions. In the production of fat yeast on a technical scale, the yield is lower, namely, 40 kg. yeast with a fat content of 18–20% as against 45 kg. normal protein yeast per 100 kg. of reducing substance of beechwood liquor.

Schmidt has discussed the nutrient requirement for fat production by yeast. *Torula* dry matter contains about 0.5% magnesium oxide, 2–3% potassium oxide, and 4% phosphorus pentoxide, the corresponding values for ash being 6%, 38%, and 50%. The magnesium content is related, according to Schmidt, to the presence of carboxylase, which is required for the conversion of  $C_3$  to  $C_2$  substances such as pyruvic acid to acetaldehyde (see Sect. VII), in the form of a "magnesium-containing diphospho-aneurin-proteid." Phosphate is involved in the conversion of  $C_6$  to  $C_3$  forms. Concerning the synthesis of fat, which we are discussing here, the following statement of Schmidt is significant:

If the phosphate supply is greatly reduced, the speed and extent of protein synthesis are reduced. This effect may be desired in certain cases, notably in

fat production. The phosphate allowance should be adjusted depending on the desired course and speed of reaction. At least 95% of the added phosphate should be taken up by the yeast. The same applies also to nitrogen supply, which should be utilized to the extent of at least 95%. Thus both loss and danger of bacterial infection are lowered to an appreciable extent.

The optimum pH for *Torula* growth appears to be 5.5. The temperature should not exceed 32–34°C.

A patent (7) may be mentioned which describes the addition of neutral salts (sodium chloride, sodium sulfate, and calcium chloride):

...process for the enrichment of the fat content of yeast during growth in aerated nutrient solutions, which consists in the addition of indifferent water-soluble substances, such as neutral salts, in quantities of at least 5% to the proper nutrients, in which the nitrogen-containing substances are lying, under the minimum amount needed for protein formation.

Yeast produced by this method is said to contain 10% fat.

*Note on Torula.* Some comment may be given on an interesting matter which is certainly more related to the synthesis of protein than that of fat.

Recently, Windisch (89) reported an extensive microbiological investigation of industrial "*Wuchshefen*." His conclusions differ widely from those of Fink, Lechner, and others. The question of xylose utilization is taken up, and, in this connection, it is suggested that the possibility of substrate adaptation has been overestimated and criticism is directed at the work of Lechner (37). According to the latter (38), the appearance of ability to utilize pentose is correlated with a change in the form of the cells (37,38). He states:

Growth of *Torula utilis* in xylose is associated with an alteration of the cell form. The alteration is only evident, however, in a widely variable fraction of the cell population. When the xylose medium is aerated, round cells, which are distinct from the characteristic, small oval cell type, are formed. Their size varies, and in some cases they may be several times as large as the normal cell. This cell type has not hitherto been known in *Torula utilis*. The round cells are produced in response to a specific environment and revert to the normal oval form in subculture.

Windisch suggests that Lechner was not in fact working at this stage with *Torula*, but with *Candida tropicalis*, which is identical with *Monilia candida* Bonorden of the Berlin Institute. He bases this view on the fact that Lechner (36) was unable in 1938 to obtain utilization of pentose or fermentation of galactose with his strain.

One year later (37), however, pentoses were in fact utilized and galactose was found fermentable. In patents applied for by Scholler (69) and Windisch (88) in the interval between these publications, priority is claimed for the discovery that *Monilia* yeasts are able to utilize pentoses. Fink (12) has recently reaffirmed the conclusions earlier reached in an investigation in collaboration with Gailer and Glaubitz (13):

The formation of giant colonies and the surface pellicle and mycelial growth habits of *Torula utilis* are subject to basic modification only by the alteration of the  $rH$  of the nutrient solution, and *Monilia*-like modifications as well as pellicle-forming types can thus be induced.

The discussion between Lechner and Windisch is still going on (38a,89a). Windisch also states that industrial protein synthesis is carried out in some cases by means of *Monilia* instead of *Torula*. Windisch mentions I. G.-Werk Wolfen-Bitterfeld, Holzverzuckerungswerke A. G. Ems (Graubünden in Switzerland), and Zellstoffwerk Attisholz (Switzerland).

### *Rhodotorula glutinis*

The marked ability of *Rhodotorula glutinis* to form fat was observed under laboratory conditions in plate as well as in submerged culture by Nilsson, Enebo, Lundin, and Myrbäck (50). The yield of crude fat was found to be 14.4% of the dry weight of the yeast in four days, whereas three species of *Torula* yielded only 5.0 to 5.3% fat. Fat formation was favored by nitrogen deficiency in a medium supplemented with extract of wheat straw (bios factors). The cells were grown in 10 liter flasks containing 1 liter of medium; aeration was effected with the help of a porous clay cylinder (Aerolite). A crop that contained 35.4% fat in the dried yeast was grown in low nitrogen medium of the following composition:

30 g. glucose, 1.0 g. ammonium hydrogen phosphate, 2.5 g. disodium hydrogen phosphate, 1.0 g. potassium sulfate, 0.5 g. magnesium sulfate ( $MgSO_4 \cdot 7H_2O$ ), 0.5 g. calcium chloride ( $CaCl_2 \cdot 6H_2O$ ), 6.0 g. citric acid, 75 g. wheat straw (extract), and water to 1 liter.

Calculated on a basis of sugar consumed (as distinct from total sugar present), the yield of fat amounted to 11.1%. After 98 hours, the consumption of sugar totaled 26.5 g., and the yield of yeast was

8.32 g. or the equivalent of 31.3 g. yeast per 100 g. sugar consumed. The fat coefficient as defined by Rippel is then 9.8. The principal difficulty of the process is considered by the Swedish investigators to be the excessive foaming. Allowing a very large head space in the fermentation vessels (10/1), this difficulty could be controlled to some extent. This method is clearly unsuitable for use with sulfite liquor. Schmidt (67) has drawn attention to a number of other drawbacks: "the process cannot be conducted well on an industrial scale, but has only academic interest, since it requires an investment of 72 parts by weight of citric acid, 900 parts by weight of wheat straw in the form of an extract, and an aeration time of 4 days for a yield of 100 parts by weight of yeast." In continuous production as worked out for beechwood liquor, Schmidt found a required aeration time of ten hours, or only twice the time requirement of protein production.

*Rhodotorula glutinis*, like *Torula*, is an imperfect fungus. Like all rhodotorulaceae, it forms a reddish pigment which bears a certain resemblance to carotene. In beer wort, the cultures form a reddish-yellow deposit of oval-shaped cells, 3.5 to 5.2 by 3.3 to 6.9  $\mu$  in dimension, and no surface pellicle. Fermentation tests are negative.

#### E. *FUSARIUM*

As has already been noted briefly in Section II, a technically feasible process of fat biosynthesis based on submerged culture of *Fusarium* has been developed by Damm (6).

Fusaria are a group of multicellular filamentous fungi. A detailed study of the group from the point of view of its phytopathology has been contributed by Wollenweber and Reinking (91). The fusaria belong to the family Tuberculariaceae of the Hyphomycetes and are distinguished by the formation of falcated conidial fruiting bodies. The diversity of the stock is matched in the multiplicity of the forms of the various species of *Fusarium*. The latter comprises sixteen groups. In thirteen of these the principal fruiting form is known. Wollenweber and Reinking differentiate 65 species, 55 varieties, and 22 forms (6). Many of the fusaria are plant pathogens. *Fusarium lini* Bolley, for example, is the causal agent of a flax blight. *F. lycopersici* causes the withering of tomatoes.

The belief entertained by Damm that little is known concerning the biochemistry of fusaria fails to take adequate account of ex-

tensive studies conducted in this field during the last ten years by Nord (51,53,54). His work, which has provided new insight into the chemical mechanism of fermentation, illuminates clearly the oft-neglected fact that cell-free enzyme systems can represent only partially the totality of reactions that occur in the living cell and need not in fact always have their counterpart in the reaction sequences of the living cell (53). Nord, Dammann, and Hofstetter (55) showed as early as 1936 that fusaria grown on surface culture



Fig. 5. *Fusarium lini* B.,  $\beta$ -form (55), grown four weeks on a glucose-starch medium ( $\times 400$ ).

possess a high fat content. They distinguish an initial  $\alpha$ -form from a  $\beta$ -form, which appears in the second or third week. The  $\beta$ -form exhibits a pronounced granulation whose properties and appearance suggest that it consists of droplets of a fatty character. Niethammer (49), too, has noted the high fat content of fusaria in experiments on mixed cultivation of *Fusarium* and *Mucor*. According to Niethammer, oil formation is most pronounced in submerged mycelial bundles and often, it is particularly intensive when the growth is inhibited in one way or another.

The technically important method of submerged cultivation of fusaria was developed by Damm (6). His earliest patent was submitted in 1937, and appeared in 1942 (24). Full data were published only in February, 1943 (6). The proliferation forms of fusaria in submerged cultivation are discussed by Damm without reference to the naturally occurring forms. "In submerged

cultivation, the form in which the organism is employed by the fermentation industry, fusaria occur only as 'fungi imperfecti.' In addition to mycelial cells whose lumen varies widely according to the nature of the growth medium and the duration of culture, only falcated and generally partitioned conidia

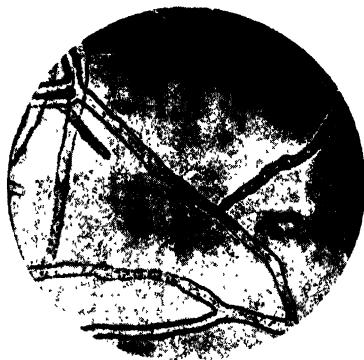


Fig. 6. Submerged mycelium showing beginning of fat formation (6).



Fig. 7. Submerged mycelium with good fat formation (6).

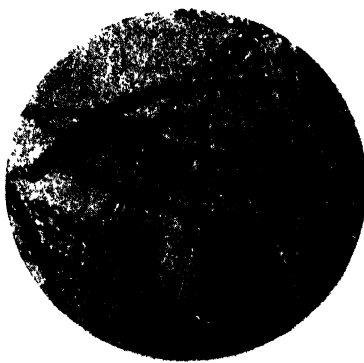


Fig. 8. Submerged mycelium with maximum fat formation (6).

and chlamydospore forms are to be found. These three types and their early stages have the diversified microscopic appearance as shown in Figures 6, 7, and 8. The mycelium occurs in submerged culture in the form of small clumps which assume beard, star, thread, and sphere shapes depending on the intensity of stirring or aeration."

In connection with the technical execution of the process, for which as in any industrial fermentation selected strains are required, Damm refers to an Italian patent as follows (25): A batch, which has been inoculated with an adapted strain of *Fusarium* and contains 5% by weight of utilizable carbohydrate and the necessary amounts of nitrogen and phosphorus compounds, is maintained in a closed vat and subjected to fine aeration at a temperature of 24–27°C. After 40–48 hours, the fat formation is optimal. The mycelium is spun down and its fat isolated. The fat content amounts to 50% on a basis of dry mycelium. The yield of dry matter is 12–15 kg. per m.<sup>3</sup> but may be raised by additions of growth substances or related compounds.

Damm notes in this connection that lower concentrations of carbohydrate had been employed more recently. A sketch of the apparatus is also presented. The principal unit consists of a tall closed fermentation vat equipped with a stirring arrangement, fine as well as coarse aeration, gas outlets, usual measuring equipment, and inlets for nutrient medium and for pure cultures. The size of the inoculum is adjusted to permit a forty- to eightyfold increase of the cell mass. The mycelium can be concentrated with the use of an ordinary centrifuge to a dry matter content of 35%.

A patent not mentioned by Damm (6), assigned to Henkel and Company (26), is of interest here although it does not concern the same microorganism. According to its title, the patent concerns a process for the cultivation of microorganisms with a view to isolation of fat and other cell constituents. The claim then continues: "representatives of the Ascomycetes and Phycomycetes groups able to grow in submerged cultures are first subjected to aeration in suitable nutrient media so as to lead to a predominance of chlamydospores and early chlamydospore stages." "The cultures are then permitted a rest period without aeration during which pellicle formation is prevented." Nutrient supply and reaction conditions are controlled to favor an extreme predominance of chlamydospores or their immediate precursors (mycelium consisting of cells of wide lumen packed with reserve substances) at the termination of the fermentation period. As examples of the organisms used, *Rhizopus oligosporus*, *Mucor racemosus*, *Mortierella pusilla*, and *Penicillium roqueforti* are named. A French patent of the same company (27) bears an analogous title: "Microorganisms of the Ascomycetes and Phycomycetes groups able to grow in submerged cultures are subjected to coarse and fine aeration in order to produce chlamydospores and their precursor stages." Maintaining the pH at 2.6 to 4.5, the yields of fat on a dry weight basis after an aeration period of two days were as follows: *Rhizopus oligosporus*, 22.8%; *Mucor racemosus*, 12%; *Mortierella pusilla*, 59.6%. Chlamydospores are formed by an asexual process, which consists in a rounding out of the parent cell, a thickening of the cell membrane, and eventual breaking off of the hyphal cells.

The nutrient requirements of *Fusaria* in submerged culture have been discussed by Damm (6). Carbohydrates, organic acids, acid amides, and other compounds may be employed as sources of carbon. Glucose and fructose are taken up at the same rate. Sucrose is presumably hydrolyzed by the fungal sucrase, but conclusive proof of this assumption has not been presented. The findings of Willstätter in yeast suggest the possibility that sucrase action may not be necessary for sucrose utilization (*cf.* 40). Other disaccharides and pentoses are taken up at a slower rate than hexoses. Fat formation occurs even at low sugar concentration (3%). This is important in the utilization of waste water.

*Fusarium* is an omnivore of nitrogenous foodstuffs. Ammonium salts, nitrates, urea, acid amides, and amino acids can all be used as sources of nitrogen.

The mineral requirements do not present any special feature. Potassium and magnesium should be supplied in abundance. Needed trace elements are generally present in adequate amount in factory water. If trace elements are provided, addition of growth substances is unnecessary, since the latter can be synthesized by the fungus itself. Nevertheless Damm renounces trace elements for economical reasons and recommends an addition of growth substances to the medium as in yeast production. The rationale of this is not evident.

Mold mycelium grown in submerged culture exhibits a higher degree of susceptibility to poisons than a yeast. A different morphological form and distinctive permeability characteristics underlie numerous differences in the behavior of *Fusarium* and *Saccharomyces*. The cell wall has a markedly different chemical composition in the two groups, mannan being dominant in the cell wall of *Saccharomyces*, whereas chitin simplexes are predominant in the mold.

The septate filamentous structure of mold mycelium contrasts sharply with the "streamlined" shape of a yeast, and imposes a ceiling on the possible density of a mold population. The operation of a limiting space factor, rather than of an inhibition due to accumulated products of metabolism, is reflected in values for mold yield per unit volume of medium, which have been cited in a previous paragraph.

The yield of fat in submerged culture corresponds to a fat coefficient of 15, as required by Ripple (see Sect. III). The pH is main-



tained between 2 and 4 "in order to avoid unnecessary oxidation of carbohydrate and formation of alcohol, which could lead to an undue decrease in the fat coefficient." The economic coefficient is much higher than is the fat coefficient. The ratio fat/protein/cell wall constituents is 1/1/1, approximately. According to a French patent, the mycelium contains 26–30% fat (25).

The fats are held by the cell tenaciously. The situation in this respect is similar to that observed in *Endomyces* by Lindner (41) and in *Oidium* by Fink *et al.* (15,67a). Special measures are necessary to effect the removal of the fat. According to a method described in a French patent (28), the material spun down from the growth medium is treated with low molecular alcohols or ketones to dehydrate the protein before extracting the fat by the usual procedure. In one example of this process, 30 kg. mycelium containing 16.9% fat and 62.2% water was treated for six to eight hours with 76 kg. of 98% methanol. The methanol removed 0.17 kg. fat. Subsequent extraction of squeezed out mycelium with diethyl ether gave 5.04 kg. fat corresponding to 26.8% of the dry weight of the mycelium. A similar method has been devised for analytical purposes by Dirr and von Soden (8).

Characteristic properties of the fat product are summarized in Table I. According to Nord, *Fusarium* oil is very similar to olive oil. Damm has asserted that the nonsaponifiable fat fraction, which Nord and Mull (56) have estimated at 2.1%, is subject to marked fluctuations depending on the procedures used. The oil has a more or less reddish color, and is supposed by Damm to contain a carotenoid pigment. Attention is directed in this connection to aurofusarin,  $C_{30}H_{20}O_{12}$ , a product derived from *Fusarium culmorum* by Ashley, Hobbs, and Raistrick (1). Nord (52,55) pointed out as early as 1936 that there is a connection between fat formation and the occurrence of pigments, but did not investigate the question further at the time. A small amount of a pigment was obtained by him from *F. oxysporum*, and a connection between this substance and aurofusarin was presumed. If the growth medium becomes alkaline, the red color of the pigment changes to blue. Recent investigations of *F. graminearum* Schwabe by Nord *et al.* (45,72) show that large amounts of rubrofusarin ( $C_{16}H_{12}O_5$ ) are formed at pH 8, whereas aurofusarin ( $C_{30}H_{20}O_{12} \cdot H_2O$ ) is formed in larger amounts

at pH 4. Rubrofusarin is either 2,8-dihydroxy-1-methoxy-7-methyl-xanthone or 2,3-dihydroxy-8-methoxy-7-methylxanthone. Recent work (57) has led to the isolation from *F. solani* D<sub>2</sub> purple of still another pigment, C<sub>15</sub>H<sub>14</sub>O<sub>6</sub>. These pigments and a number of synthetic xanthenes influence growth and dehydration of alcohol by fusaria. Minimal concentrations (250 µg. per liter) of the *F. solani*

TABLE I  
CHARACTERISTICS OF FATS DERIVED FROM MICROORGANISMS

Source and ref. no.	Solidi- fication temp., °C.	M.P., °C.	Sapon. no.	Acid no.	Iodine no.	Ester no.
<i>Penicillium javanicum</i> (87)	6-7	15	191-192	10.5	84	...
<i>Aspergillus niger</i> (3)	...	...	169	71.2	95.1	98
<i>Endomyces vernalis</i> (see 21b)	-10	...	185	13.0	96	...
<i>Endomyces vernalis</i> (21b)	solid	...	238	...	65	...
<i>Endomyces vernalis</i> (21b)	liquid	...	229	...	105	...
<i>Oidium lactis</i> (67)	...	...	...	...	63	...
<i>Oidium lactis</i> Stamm A (15,67a)	...	...	193.8	6.11	46.8	...
<i>Oidium lactis</i> Stamm B (15,67a)	...	...	198.0	4.53	72.3	...
<i>Fusarium</i> (55)	liquid	...	189.3	...	84.7	...
<i>Fusarium</i> (6)	liquid	...	190-196	0.5-4	79-90	192
<i>Torula utilis</i> (69a)	...	...	180.7	102.4	120.5	78.3 <sup>a</sup>
Yeast (see 55)	...	...	162	?	71.1 <sup>f</sup>	...
Peanut oil	...	...	183-197	...	89-98	...
Olive oil	<-6°	...	182-188	...	87-88	...

<sup>a</sup> 30% free oleic acid.

<sup>b</sup> 23% free oleic acid.

<sup>c</sup> 1% nonsaponifiable matter.

<sup>d</sup> 2.1% nonsaponifiable matter.

<sup>e</sup> 12.3% nonsaponifiable matter (including 53% ergosterol, 25% squalene, and 10% C<sub>15</sub>H<sub>14</sub>O<sub>6</sub>).

<sup>f</sup> 15.6% nonsaponifiable matter.

pigment, for example, reduce the dehydration of isopropyl alcohol by 12%. Nord *et al.* (57) summarize the work on the connection between carbohydrate assimilation, fat formation, and the occurrence of the pigment as follows:

"Since both *Fusarium lini* Bolley as well as *Fusarium lycopersici* are powerful fat-forming molds, experiments were carried out to investigate a possible influence of the pigment on the relationship between carbohydrate consumption and fat production. When *Fusarium solani* D<sub>2</sub> purple is grown on an acetate instead of carbohydrate containing Czapek-Dox medium, the

TABLE II

Condition	<i>F. lini</i>	<i>F. lycopersici</i>	<i>F. solani</i>
RAULIN-THOM MEDIUM			
Pigment formation	None	Increases with increasing (2.5, 5.0, 10.0%) glucose concn.	Max. fat prodn. coincides with max. pigment formation (maxima being on 5% glucose)
Fat formation	++	Max. fat prodn. coincides with max. pigment formation	
Solanione addn. (1 mg. per l. medium)	50% decrease in mycelial wt.; carbohydrate conversion factor lowered; dehydrogenation of isopropanol lowered; fat contains more desatd. fatty acids (iod. no. 138 instead of 84)	No influence because of abundance of pigments in the mold itself	
CZAPPEK-DOX MEDIUM			
Pigment formation	None	None	Max. fat prodn.; highest carbohydrate conversion factor; accompanied by max. pigment prodn. (maxima being on 10% glucose)
Fat formation	++	Decreases with increasing (2.5, 5.0, 10.0%) glucose concn.	

pigment formation is almost suppressed, and the fat formation is increased. When *Fusarium lini* Bolley is grown on a Raulin-Thom medium containing 2.5, 5, and 10% of glucose, to which has been added 1-4 mg. of pigment, it was found that in all cases the growth of *Fusarium lini* Bolley is suppressed about 50%. However, the presence of the pigment causes an increase in the extent of fat formation at different concentration levels of glucose."

Nord and co-workers (6a,15a,56a) observed in further investigations:

"...that when solanione...is added to a growing culture of the nonpigment producer *Fusarium lini* Bolley, there is a decrease in mycelial weights, and the carbohydrate conversion factor is greatly lowered. *Fusarium lycopersici*, on the other hand, produces fat and pigments to a larger extent when grown on Raulin-Thom media containing increasing amounts of glucose. Similarly, the mold *Fusarium solani* D<sub>2</sub> purple was shown to have a maximum lipide production accompanied by maximum pigment accumulation when the glucose concentration amounts to 5% in the Czapek-Dox media."

These and other observations are summarized in Table II.

Deschamps (6a) recently found a very interesting influence of the pigment solanione on the type of fat produced by the molds. Addition of solanione to Raulin-Thom medium employed for the growth of non-pigment producer *F. lini* Bolley increases the desaturation of fats produced. The iodine number changes from 84 to 138, which can only be due to an increase in linoleic acid, established by measurement of the ultraviolet absorption at 628 m $\mu$ . On the other hand, solanione has no appreciable effect on the growth of, or type of fat produced by, *F. lycopersici*, which can be cultivated as a pigment producer on Raulin-Thom medium. In the case of *F. lini*, the pigment seems to affect the hydrogen transport system present in this mold.

## VI. Recovery and Properties of Fat Products

The term "fat" will be used throughout this section to designate the crude fat fraction that can be removed by ordinary extractants and consists of a mixture of glycerol esters of fatty acids, phosphatides, sterols, etc.

Isolation of fat from microorganisms presents a number of difficulties. Complete extraction without previous destruction of the cell structure is hindered by the binding of the fat to proteins (76). To overcome the resistance to extraction, Lindner proposed three methods for use with *Endomyces*: (1) grinding of the dried mycelium with sand and subsequent extraction with ether; (2) treatment with dilute hydrochloric acid (unlocking effect); and (3) autolysis at 50°C. The first procedure entails decomposition to an extent of 25% of the fat, and yields a product that can be used only for technical purposes. The others lead to edible fat and to a by-product protein that can have many uses, e.g., as a supplement in food condiments.

In the method described by Henkel and Company (see Sect. VE), mycelium of fusaria is treated with alcohol or acetone to denature the proteins. Extraction of the fat is thereby made feasible. Schmidt of Mannheim-Waldhof offers no information concerning extraction of fat from *Torula*.

The difficulties encountered in technical isolation are of course also met in analyses of fat of microorganisms. Haehn and Kintoff (21) have relied on alkali to unlock the fat (according to the procedure of Stockhausen and Ericson) and determined the isolated fatty acids by titration with alkali. According to Reichel (62), the error in this case is 8%. Fink considers the method inadequate. Reichel and Reinmuth (64) avoid the alkali unlocking step as follows: the mycelium is dried to constant weight *in vacuo* at 50–70°C., and, after being ground with quartz sand, is extracted for six to eight hours with petroleum ether. The solvent is allowed to evaporate and the residue taken up in petroleum ether. The alkali equivalent of the resulting solution is determined before and after exposure to the action of lipase of castor bean. The amount of fat is estimated from the difference of the two values.

Fink *et al.* (15,67a) ground mycelium of *Endomyces* and *Oidium*, which had been dried at 105°C., in a ball mill for three hours, added quartz sand, and ground the mixture for another fifteen minutes, finally extracting for six hours with ether in a Schmalfuss extractor. Extraction was continued until the yield of fat in the last batch of solvent was not more than 0.3%. The pooled extracts were evaporated and the residual fat dried to constant weight at 80°C.

Nilsson *et al.* (50) released the fat of *Rhodotorula* by boiling in hydrochloric acid, adsorbed the fat thus freed on cellulose, and extracted the adsorbed fat with ether after drying. Dirr and von Soden extracted with ether after preliminary treatment with alcohol.

The fat products have often been said to be similar to olive oil, and this description is to some extent also borne out by the iodine and saponification numbers. Paul considers this comparison to be misleading in view of the tendency of microbe fats to become rancid. As is evident from the values assembled in Table I, the free acid content of several microbe fats is rather high. Reichel (62) has found that fat of *Endomyces* grown on glucose, fructose, or sucrose contains 5–8% free fatty acids and 7–12% neutral fat. It seems reasonable to consider that the dependence of fat composition on the

conditions of growth, as demonstrated by Damm (6) for the case of fusaria, applies also in other cases.

Among the individual fatty acids, oleic acid has been found to be a major constituent in those fats for which a detailed analysis is available. Linoleic acid was found present in relatively small amount, whereas little or no linolenic acid could be detected. Of the saturated fatty acids, stearic and palmitic acids are present, in addition to smaller amounts of lower homologs. The fat of *Aspergillus* and *Penicillium* contains also some lignoceric acid (tetracosanic acid), which does not occur in yeast fat.

According to Ward *et al.* (87), fat of *Penicillium* contains 30.8% saturated and 60.8% unsaturated fatty acids. The fatty acid mixture is of the following composition: 31.1% oleic acid, 29.1% linoleic acid, 0% linolenic acid, 21.4% palmitic acid, 8.6% stearic acid, and 0.8% lignoceric acid.

According to Schmidt and Kaufmann (68), fat of *Oidium lactis* contains 42.8% saturated and 53.1% unsaturated fatty acids. The fatty acid mixture contains 25.7% palmitic acid, 17.1% stearic acid, 41.2% oleic acid, and 11.8% linoleic acid. Hydroxy acids account for 3.7% and linolenic acid for 0.12% of the total fatty acids.

Reichert (64a) extracted with warm methanol and diethyl ether *Torula utilis* (Cellulosefabrik Attisholz) which had been dried on drums after plasmolysis to a water content of 6.9%. He found 6.40% lipides (dry weight basis). Extraction with ether alone gave too low values. 59.9% of the lipides were soluble in acetone, while 37.7% phosphatides remained undissolved. The characteristics of the fat are given in Table I. Especially noteworthy is the extremely high acid number: 102.4. The fat contained 77.3% fatty acids, 12.3% unsaponifiable matter, and 5.8% glycerin. The fatty acids were distributed as follows: 12.2% saturated fatty acid (2.3% C<sub>14</sub> and lower, 64.4% palmitic, 31.1% stearic, and 1.9% C<sub>20</sub> and higher); 1.2% solid unsaturated fatty acids; and 87.7% liquid fatty acids. The latter contained 5.0% linolenic, 56.7% linoic, 24.5% oleic, 8.6% palmitic, and 5.2% liquid saturated fatty acids. The linoic acid content was much higher than in other fats from microorganisms. Palmitic acid has not yet been found by any investigator.

Little information is available concerning the amounts of phos-

phatides, sterols, and other constituents that may be present (*cf.* 3,15a).

Fink *et al.* (14) have discussed the nutritional value of *Endomyces* fat on the basis of experiments conducted by Voltz. It is considered on the basis of these experiments that 60% of the dried mycelium consists of digestible organic matter, which has the following constituents: crude protein, 14%; fat, 19%; and carbohydrate, 26%. Of the extracted fats, 87% is digestible. *Fusaria* grown in surface culture according to the procedures of Nord and collaborators have been found to be equivalent to brewer's yeast as a source of B vitamins provided that thiamine is added (85). Mycelial residues of Damm's process contain, according to Kapfhammer (33), the following: arginine (3.4%), proline, hydroxyproline, lysine, histidine (0.5%), tyrosine (2.2%), and leucine. This product is inadequate as a sole source of protein in the diet but forms a valuable supplement.

Chitin, which is present in the cell wall of *fusaria* in the amount of 45–55%, can be recovered from the defatted mycelium by a method described in a French patent of Henkel and Company (29). The procedure involves treatment with water and removal of albumin with 1% sodium hydroxide.

## VII. Chemistry of Fat Formation

Various aspects of fat metabolism and of its relationship to carbohydrate metabolism have been reviewed in preceding volumes of this series (5,56,82,90). The synthesis of fat by microorganisms has been discussed cursorily by Clifton and Nord. The present summary is based on several recent research papers of German workers and on reviews by Fink *et al.* (14), Bernhauer (3), Smedley-Maclean (76), and Hesse (30). Although many details remain to be filled in, the following appear now to be established facts: (a) microbe fats are synthesized from carbohydrates, just as are plant and animal fats; (b) glycerol is formed as in alcoholic fermentation; (c) synthesis of neutral fat from fatty acids and glycerol involves the activity of lipase. Relatively little is known concerning the mode of synthesis of constituents that accompany the fats, *i.e.*, phosphatides, sterols, and carotenoids.

Contrary to the view of Emil Fischer that fatty acids are synthesized by direct condensation of numerous sugar molecules (*e.g.*,

stearic acid from three molecules of hexose, palmitic acid from one molecule of hexose and two molecules of pentose), the view has been widely accepted that fatty acids are built up from  $C_3$  or  $C_2$  fragments derived from sugars by desmolysis. The participation of a  $C_3$  unit, especially that of pyruvic acid, in fatty acid synthesis has been supported by the work of Smedley-Maclean. Recently, she too has tended to favor a view that she preferred in 1912 and that was accepted for the case of butyric acid fermentations by Buchner and Meisenheimer for fat synthesis by *Endomyces*. (This view was also accepted for liver autolysis by Magnus-Levy, 1902, and in 1925 by Haehn and Kintoff, 21.) According to this theory, acetaldehyde is the key substance of the synthesis, and acetaldehyde condensation to aldol the first step of the process (cf. 16). The theory stems from the finding that fat synthesis by *Endomyces vernalis* is accompanied by formation of large quantities of carbon dioxide corresponding to 35–40% or more of the sugar. This amount of carbon dioxide cannot be accounted for by oxidative respiration alone; the conclusion that carboxylase activity is involved appears to be justified. Formation of pyruvic acid probably occurs by pathways followed in alcoholic fermentation. The acetaldehyde formed from pyruvic acid by the action of carboxylase is not reduced, however, in this case to ethyl alcohol. The latter has never been detected in fat synthesis. The formation of acetaldehyde has been established by trapping it with bisulfite according to the method of Neuberger and Reinfurth and of Neuberger and Nord. Bisulfite has also been shown to reduce synthesis of fat from acetaldehyde (21), alcohols, and acetate (43). Use of acetaldehyde for fat synthesis was investigated on a technical scale by Lindner and Unger (43) and Ehrlich (9). Sonderhoff (78) has directed attention to the danger of an error inherent in exclusive dependence on trapping procedures, and has suggested that the effects of bisulfite may be related to a general inhibitory action. Haehn and Kintoff (21), however, used acetaldehyde in concentrations (up to 1%) that do not markedly retard growth. In mineral nutrient solutions containing sucrose, the yield of acetaldehyde was found to increase markedly with increasing bisulfite concentration (0.25 to 0.5 to 1.0%). It should be borne in mind that acetaldehyde bound by bisulfite might nevertheless be convertible to fat.

The above view is in agreement with balance sheet experiments

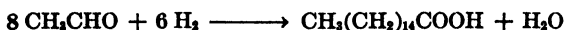


of Haehn and Kintoff (21). *Endomyces* cells used were of a protein generation (see Sect. II) taken at a growth stage when formation of mycelium was virtually at an end and sugar and acetaldehyde served almost exclusively for fat synthesis. When the nutrient medium under the *Endomyces* pellicle was replaced by a fresh batch of nutrient, recovery was 40–50% CO<sub>2</sub> and 30% fat. On replacing the original medium by a solution of 4% CH<sub>3</sub>CHO in water, the fat yield was 15%, while tap water alone yielded 8%. The quantitative data for the developing protein generation are quite different, since at this stage mycelial growth and protein synthesis proceed concurrently with fat synthesis. Raaf (61) considers nevertheless that acetaldehyde is the intermediary of both protein and fat synthesis during this stage as well. The fat coefficient, therefore, cannot be expected to exceed 15.

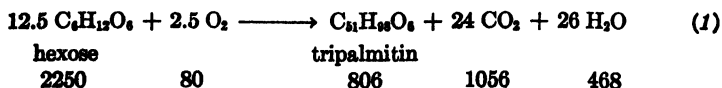
Haehn and Kintoff (21) have proposed a modified form of the Gay-Lussac equation for fermentation to describe the sequence preceding fat formation:

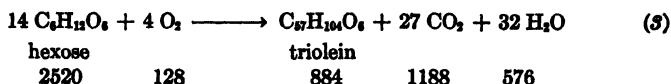


The theoretical yield of CO<sub>2</sub> here is the same as required by the equation for alcoholic fermentation, *i.e.*, 49%. The hydrogen derived from oxidation of glyceraldehyde phosphate (see scheme of Sumner and Somers, 83) is not employed as in the "classical" fermentation cycle for reduction of acetaldehyde, but is used to effect the reduction of polyene aldehydes and acids formed as the result of aldol condensation. The formation of palmitic acid from acetaldehyde and glucose may be described according to Bernhauer (3) by the following equations:

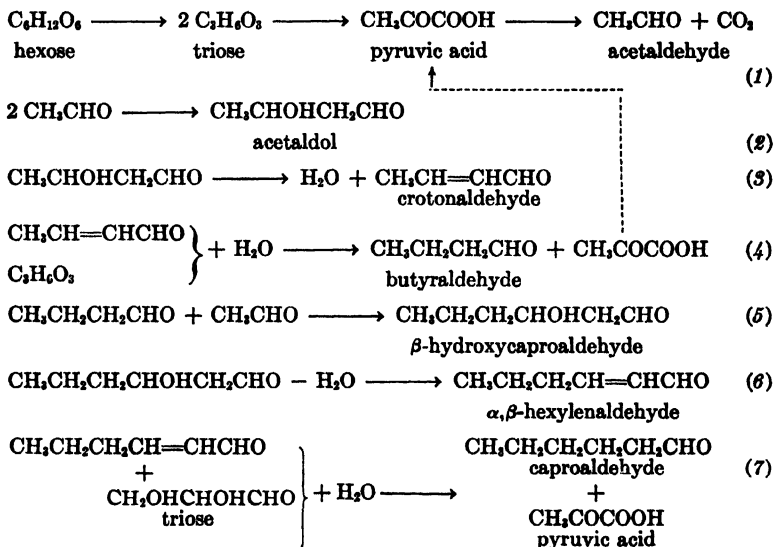


Synthesis of the most important of the triglycerides of microbe fat may be described according to Bernhauer (3) as follows:





The equations require the following theoretical yields: (1) 35.8% palmitin and 46.9% carbon dioxide; (2) 35.3% tristearin and 47.1% carbon dioxide; (3) 35.0% triolein and 47.1% carbon dioxide. Theoretical values were in fact closely approached in the experiments reported by Haehn and Kintoff. Their method of fat estimation has, however, been criticized by Reichel (see Sect. VI). The following scheme is that proposed by Haehn and Kintoff (21):

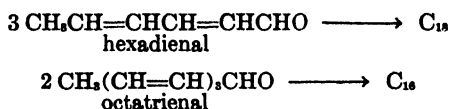


Once caproaldehyde is formed, further condensation may be assumed to occur by reaction with acetaldehyde. This is a more probable reaction, according to Haehn, than condensation of three molecules of caproaldehyde or of his unsaturated analog, the aldehyde of sorbinic acid, that is, hexadienal ( $\text{CH}_3\text{CH}=\text{CHCH}=\text{CHCHO}$ ). Reaction stages beyond aldol, it should be noted, have not as yet been verified experimentally.

The above scheme has been supported to some extent by findings of Günther and Bonhoeffer (20) with heavy water,  $\text{D}_2\text{O}$ . According

to calculations of Fink, who supplied the mycelial material for this study, 60% of the hydrogen present in the fat could have been derived from water. The value found by Bonhoeffer by extrapolation to  $D_2O$  of 100% was in fair agreement with this figure. The fungus failed to grow on 80%  $D_2O$ ; growth and fat formation were found after three weeks on 20%  $D_2O$ , and after two to three months on 50%  $D_2O$ .

Kluyver (cited by Clifton) has accepted Haehn's views with slight modifications: "In either case acetaldehyde, or some compound derivable therefrom, could be regarded as the precursor of butyric acid." Haehn and Kintoff obtained the same results with aldol, pyruvic acid, and alcohol as with acetaldehyde. Important contributions to the clarification of this problem have been made by Reichel (62,63). Assimilation tests with high saturated and unsaturated aldehydes in the form of their bisulfite addition products at concentrations of 0.1 to 0.005% were carried out on *Endomyces* and gave the following results: High saturated aldehydes (octyl and decyl but not hexyl) were oxidized to the corresponding fatty acid in five to seven days. Unsaturated aldehydes, on the other hand, were oxidized to fatty acids containing a larger number of carbon atoms, for example:



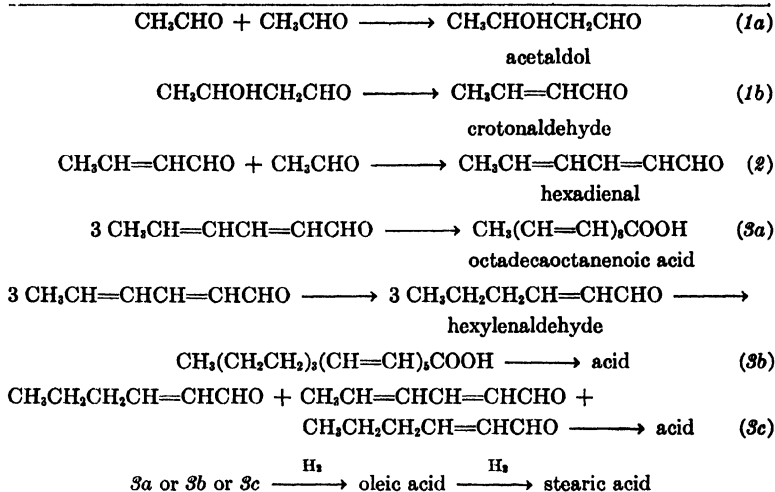
Crotonaldehyde, as well as acetaldol, by condensation of four molecules leads to  $C_{16}$ . The polyene aldehydes that are then probably formed by loss of water are converted into the corresponding fatty acid by the oxidation of the aldehyde group and the hydrogenation of the double bond.

The condensation of crotonaldehyde,  $\text{CH}_3\text{CH}=\text{CHCHO}$ , to octatrienal and to the red hexadecaheptaenal,  $\text{CH}_3(\text{CH}=\text{CH})_5\text{CHO}$ , with piperidine acetate as catalyst has been described by Kuhn (35a) in studies on the synthesis of polyenes and the structure of carotenoids. Hexadecaheptaenal leads by way of cetyl alcohol,  $\text{CH}_3(\text{CH}_2)_{14}\text{CH}_2\text{OH}$  to palmitic acid,  $\text{CH}_3(\text{CH}_2)_{14}\text{COOH}$ .

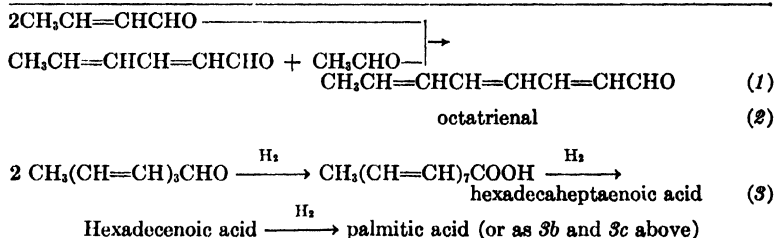
Reichel and Schmid (62,63), too, assign a key role to acetaldehyde in their scheme (Scheme I) of synthesis of the higher fatty acids.

## SCHEME I

## A. STEARIC ACID



## B. PALMITIC ACID



Its important features are that the synthesis starts from acetaldehyde and involves the intermediary formation of polyene aldehydes with an even number of C atoms. The way in which intermediary condensation steps combine with each other determines the kind of product obtained. It is important to note that fat synthesis represents a large accumulation of energy for the cell. The calorific value of fat relative to glucose is 2.5, whereas the corresponding value for protein is 1.5.

Utilization of pure glycerol as a substrate for fat synthesis has been reported by Haehn and Kintoff (21) but has been denied by

Reichel and Schmid (63). The following were found to be utilized, in mixtures with glycerol: pyruvic acid, acetaldehyde, hexadienal, and octatrienal.

Specific information concerning the mode of formation of glycerol is not available but it is generally considered that the mechanism resembles that operative in alcoholic fermentation.

Synthesis of glycerol esters is assumed to be mediated by the same lipases that hydrolyze them. Reichel and Schmid (62,63) obtained glycerides as well as free fatty acids from glycerol and pyruvic acid or from glycerol and octatrienal. It is not evident whether the excess of fatty acid in microbe fat is due to a disturbance of the synthesis or to deficiency of glycerol. No detailed study of the influence of the different components of the system glycerol-fatty acid-fat-water has been reported. It seems proper to assume, however, according to Reichel, that the effect of the different components causing a shift of the equilibrium varies with their relative concentration. Hence, glycerol excess should bring about greater synthesis, and excess of water should favor hydrolysis.

In this connection the report of Popowa and Putschkova (58a) (see also Sect. VB) on *Oidium lactis* is interesting. These investigators found that oxidative means, *e.g.*, hydroperoxide or potassium bromate, inhibit the lipase and increase the formation of fat. The inhibition obviously is concerned only with the hydrolyzing and not the synthesizing action of the enzyme.

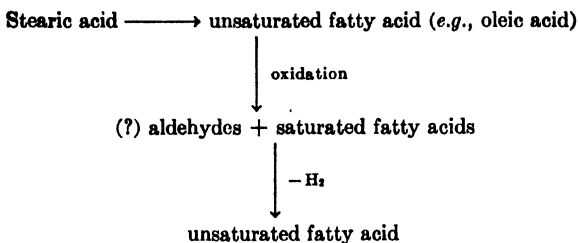
In addition to the lipases, Bernhauer (3) believes that the following are involved: enzymes that split the sugar molecule as in alcoholic fermentation to  $C_3$  forms, and cocarboxylase (the pyrophosphate ester of vitamin  $B_1$ ) whose addition has in fact been found to favor fat formation in the case of *Torula utilis*. According to Reichel, condensation of aldehyde is probably mediated by aldolase, and high polyene aldehydes that result from removal of the elements of water are converted by autoxidation or by dehydrogenation with the help of aldehyde systems to fatty acids. Hydrogenations can be effected with the help of the hydrogen that would have served in alcoholic fermentation for the reduction of acetaldehyde. In this phase, the terminal oxidative conversion of aldehyde groups to carboxylic acid groups seems also to begin.

It is important to emphasize that the results discussed above were all obtained in experiments carried out on *Endomyces vernalis*. Their validity for the synthesis effected by other species of microorganisms is undetermined. The extension of the experimental work to other species is beset by difficulties. Fink *et al.* (14) have commented on the relative ease with which studies of the mechanism of fat synthesis can be carried out in the case of *Endomyces vernalis* and certain other molds, and the difficulties that beset similar attempts in bacteria and yeasts. In the latter groups only two carbon types that favor fat formation have been identified, namely, alcohol and acetate. Wieland and Wille (87a) have found in aerobic dehydrogenation by "starved" yeast that for eleven molecules of alcohol oxidized, two are converted to fat, and one to carbohydrate.

Schmidt considers that the mechanisms of synthesis formulated by Haehn and Reichel are equally operative in the case of *T. utilis*.

The enzymic mechanism of fat synthesis in fusaria has been studied in the case of *Fusarium lini* Bolley and *F. graminearum* Schwabe by Mull and Nord (46,56). Participation of a lipase, whose existence has been shown to be probable, is a reasonable assumption. As is seen in Table I, the fat consists in this case mainly of unsaturated fatty acids. To obtain information concerning the mechanism of fat formation, experiments were carried out in which the fungus was grown in surface culture on media that contained added fatty acid or olive oil in addition to the ordinary mineral components. To stabilize the emulsion, 0.05% of polyvinyl alcohol was added. The latter is a more efficient stabilizer than either gum arabic or gum of carob bean. The progress of dehydrogenation was followed by measuring the variation of the iodine number of the fatty acids extracted from the medium. The results obtained have been summarized (56) as follows:

"The study of an enzymic action of *Fusarium lini* Bolley upon a likely precursor, such as stearic acid, indicates that the saturated acid may be dehydrogenated by an enzyme system similar to that by which it was formed in *F. graminearum*, perhaps giving rise to aldehydes (generally expressed), in accordance with the findings of Feulgen and Bersin, and to a saturated acid. The saturated acid thus obtained can be further acted upon by the enzyme system present:



Thus presumably, the precursor of the end product formed, perhaps, in agreement with the postulation of Witzemann, is not a static entity but in reality undergoes a series of changes, thereby playing an integral role in the total metabolism of the organism."

The increase in free fatty acid found in the presence of olive oil supports the assumption of the presence of lipase. It is evident as well that a fatty acid dehydrogenase is actively present.

Information concerning technical utilization of fusaria in the United States has been presented by Nord and Mull (56). Use of fusaria has been found advantageous for fermentation of sulfite waste liquors or of wheat and wood hydrolyzates and for the decomposition of lignins in processes that utilize the ability of fusaria to ferment pentoses (*cf.* 51,73). Mention was not made in this connection of technical exploitations of the fat-forming ability of the species.

### VIII. Conclusion

A discussion of technical studies is incomplete without an examination of the economic aspects. However, in the work presented above, this has been discussed only with regard to the processes of surface cultivation, which are not useful on a technical scale. They are uneconomical and are at best used in times of need. Concerning the economic success of the process of Mannheim-Waldhof (Schmidt) and that of Henkel et Cie. (Damm) nothing is known. On the other hand, it has been established that protein synthesis (to be treated in another volume of this series) by microorganisms results in a product hardly competing in price with cheap plant protein (*e.g.*, soybean). It is noteworthy that such biosyntheses nevertheless are also carried out in countries outside Germany. For these syntheses waste water is generally the substrate. It is conceivable that in

competition with the processes of biological purification of the waste waters comprising the formation of methane the biosynthetic methods may be economical. This remains for the future to show.

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### Addendum

Problems of biological fat synthesis and of fat metabolism are treated in Volume VIII of this series by Breusch, Bergström, Holman and Kleinzeller. These reviews do not touch on the industrial side of the problem, but investigators interested in the industrial biosynthesis of fat will find remarkable observations in the review of Kleinzeller (34a). His results, as well as those of Enebo *et al.*, reported by Kleinzeller, are of special interest. The Swedish authors, whose paper was not available in Germany, report fat coefficients as high as 18, with the fat content of the yeast *Rhodotorula gracilis* amounting to 60%. This value exceeds the theoretical value of Rippel (see Sect. VD), and Enebo states "that Rippel in his calculations did not expect such low protein content in the microorganisms when the fat content reaches such high figures." These results seem to be of great significance in the further evolution of biological fat synthesis.

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